3.8 Binding of SV to transforming growth factor-β receptor II

When PLA was performed using rabbit polyclonal anti-TβRII and mouse monoclonal anti-HA antibodies for the isolated fibroblasts treated with SV-HA peptide, PLA-positive red signals were found (*Figure 4C[a]*). In contrast, PLA-positive red signals were not detected in the isolated fibroblasts treated with SV-HA random peptide (*Figure 4C[b]*). We assessed the ability of SV to bind to TβRII, using a sensor chip immobilized with biotinylated SV ($K_D = 13.5$ nM), and this peptide bound to TβRII with high affinity (500 Resonance Unit; *Figure 4D*). However, SV random peptides ($K_D = 16$ nM) had a much lower R_{max} (R_{max} = analyte molecular weight (MW)/ligand MW × the immobilization level × the stoichiometric ratio) value (200 Resonance Unit).

3.9 The effects of SV on Smad activation

Treatment with TGF-β1 or SV induced the phosphorylation of TβRI, Smad2, and Smad3 to similar degrees (*Figure 4E*). Conversely, treatment with SV random peptide had no effect on TβRI, Smad2, and Smad3 phosphorylation.

4. Discussion

In this study, we transplanted myoblast sheets to the myocardium in an infarcted rat model. The cell sheets are removed from special temperature-responsive dishes without destroying the cell–cell or cell–extracellular matrix adhesions in the cell sheet. The myoblast sheet does not require an artificial scaffold, because it has a great ability to integrate with the infarcted area via an adhesion factor, such as integrin-$\alpha_7\beta_1$ and α-dystroglycan, which are expressed on the surface of myoblasts; thus, the sheets do not fall off after the chest is closed.[5–7]

The effect of myoblast sheet transplantation is mediated mainly by paracrine growth factors that stimulate the injured myocardium.[6,7] The paracrine effectors include HGF, VEGF, and stromal-derived factor 1. These factors can promote angiogenesis in the ischaemic myocardium. Hepatocyte growth factor is also associated with anti-fibrosis and anti-apoptosis. The grafted myoblasts beneficially attract haematopoietic stem cells to home in on the infarcted heart area for heart regeneration and angiogenesis by stromal-derived factor 1.[6] These paracrine activities induce angiogenesis and reduce fibrosis and hypertrophy; as a result, the depressed cardiac function improves. Therefore, we hypothesized that functional modification of myoblast sheet properties by overexpressing a factor associated with angiogenesis, anti-fibrosis, and anti-apoptosis could further promote and maintain the therapeutic effects of the sheet. Our previous results demonstrated that SV has a much stronger pro-angiogenic action than VEGF.[14] Given that SV has a straight-chain sequence, rather than a complicated conformation, we can speculate that this peptide would be degraded by peptidase within an organism. Our previous research has shown that synthetic SV has no effect on the proliferation of endothelial and muscle cells.[13,14] The degradation rate and function for the proliferation of SV could have high biocompatibility with peptides. In this study, we investigated the effects of SV-secreting myoblast sheets in infarcted rat hearts.

Most of the transplanted myoblasts drop out at 4 weeks after sheet transplantation.[21] As a result, cardiac function in the WT-rSkM group at 4 weeks after sheet transplantation was markedly decreased. In contrast, in the SV-rSkM group the functional improvements were maintained for 8 weeks after sheet transplantation. The capillary density 8 weeks after transplantation was significantly higher in the SV-rSkM group than in the control and WT-rSkM groups. The vessels newly formed by the secreted SVs from the myoblast sheets remained until 8 weeks post-transplantation, after the drop-out of the transplanted cells. The paracrine factors from transplanted myoblasts also promoted angiogenesis. Thus, in this study, the secreted SV showed an enhanced angiogenic action after myoblast transplantation. It is possible that SV induced angiogenesis in both the surviving cardiomyocytes and the transplanted cells; as a result, the survival time of the transplanted cells would have been extended. However, there are no data concerning the effect of SV-rSkM on the endogenous mobilization/proliferation/apoptosis and differentiation of cardiac resident cardiac stem/progenitor cells. More research is needed to define the effects of SV on these cells.

Siltanen *et al.*[11] reported the efficacy of a heart failure treatment involving the transplantation of myoblasts genetically modified to overexpress HGF. Hepatocyte growth factor is a cardioprotective factor associated with angiogenesis, anti-fibrosis, and anti-apoptosis.[22,23] Hepatocyte growth factor-overexpressing myoblast sheets stimulated angiogenesis and inhibited myocardial fibrosis in a rat chronic heart failure model. However, cardiac function was not improved by the transplantation of HGF-overexpressing sheets.[16] In contrast, SV-expressing sheets, which also have a pro-angiogenic action, enhanced cardiac function and angiogenesis. Transplantation of SV-secreting sheets enhanced the functional recovery of ischaemic myocardium compared with the findings in the control and WT-rSkM groups. In particular, systolic parameters, such as LVIDs and ESV, were significantly improved in the SV-rSkM group.

Myofibroblasts share morphological features with fibroblasts and smooth muscle cells. Differentiated myofibroblasts are characterized by increased α-SMA and the morphological features of well-developed stress fibres.[24] Although myofibroblasts in normal tissue, granulation tissue, and pathological tissue exhibit phenotypic α-SMA expression, SM-MHC, vimentin, and desmin, myofibroblasts more commonly express α-SMA.[25] Myofibroblasts have a greater contractile capability than undifferentiated CFs, and this property is believed to be important in maintaining the structural integrity of healing scars.[26] Expression of α-SMA in stress fibres is instrumental in force generation by myofibroblasts.[27] Additionally, myofibroblasts confer mechanical tension to remodelling matrix via anchoring and contracting.[24] In this study, many clusters of SMA-positive and SM-MHC type 2-positive cells were observed in infarcted areas in the SV-rSkM group. These cells differentiated from CFs into myofibroblasts in the infarcted area after the addition of SV, and the myocardial contractile performance of the infarcted wall in the SV-rSkM group was improved by the accumulation of myofibroblasts. Our previous study indicated that, when skeletal myoblast sheets were transplanted into a swine acute MI model, well-developed smooth muscle cells accumulated in the centre of the scar.[28] In our study, more SMA-positive cells accumulated in the infarcted area in the SV-rSkM group than in the WT-rSkM-group, and the secreted SV enhanced the effect of SMA expression by CFs. Furthermore, owing to the accumulation of myofibroblasts in the infarcted area, adverse effects on the uninjured myocardium and its exercise endurance were decreased; consequently, cardiac remodelling processes, such as fibrosis and cardiomyocyte hypertrophy, were attenuated. The fibroblasts in scar tissue of the infarcted area are differentiated into SMA-positive and SM-MHC type 2-positive cells by SV. There is no cell–cell connectivity between these cells and the recipient's cardiomyocytes, and it is possible that they have not been synchronized with the cardiomyocytes. However, they do have a contractile capability, and SV could have transferred the contractility to the infarcted wall via the

accumulation of these cells, improving the motion of the scared left ventricular wall and inhibiting the dilatation of the LV chamber in the SV-rSkM group.

Our previous research has shown that synthetic SVVYGLR peptides *in vitro* activate the adhesion and migration of endothelial cells and smooth muscle cells, and stimulate tube formation by vascular endothelial cells.[13,14] In contrast, SV has no effect on the proliferation of these cells, whereas it enhances the adhesion and proliferation of several types of human mesenchymal cells.[17] Although the effects of SV on apoptosis in these cells have not been evaluated, the results regarding proliferation suggest that SV has no effect on apoptosis. According to these data, SV should have no impact on the proliferation and apoptosis of myoblasts, while stimulating the proliferation of fibroblasts and myofibroblasts.

Osteopontin is highly expressed during the differentiation of fibroblasts into myofibroblasts, and could have an effect on fibroblast differentiation and a role in myofibroblast function during tissue remodelling.[29] Transforming growth factor-β plays an important role in the activation of fibroblasts in wound repair, and it induces myofibroblast differentiation via Smad signalling.[30] Osteopontin is required for the differentiation and activation of myofibroblasts formed in response to TGF-β1.[31] This study illustrated that, in isolated CFs, SV had a great degree of affinity for TβRII and activated Smad signalling via TβRs. The secreted SV bound TβRII and induced the differentiation of fibroblasts into myofibroblasts through TGF-β receptor–Smad signalling.

Transforming growth factor-β participates in vascular development and the maintenance of vascular homeostasis, and it induces angiogenesis at low levels.[32] Transforming growth factor-β regulates angiogenesis by acting on both vascular endothelial and smooth muscle cells.[31] SV also stimulates angiogenesis at low levels, but this effect plateaus at high levels.[14] Thus, SV induces angiogenesis via the same mechanism as TGF-β. However, we believed that SV could also bind receptors other than TβRII and exhibit myocardium-protecting actions, such as promoting angiogenesis and inhibiting hypertrophy. To explain the effect of SV in improving cardiac function, SV receptors in myocardial tissue will have to be identified, and the details of its mechanism will need to be examined.

Functional SV peptide-secreting myoblast sheets facilitate long-term improvement in cardiac function and inhibition of cardiac remodelling. The SVs secreted from myoblast sheets effectively stimulated angiogenesis in the failing myocardium. The accumulation of SMA-positive cells induced by SV confers a contractile property on the infarcted wall. The early therapeutic effects after SV-secreting myoblast sheet transplantation were due to the paracrine effects of the transplanted myoblasts, and the late effects were caused by the pro-angiogenic effects of SV and its induction of myofibroblast accumulation via TGF-β–Smad signalling. These results suggest that SV could change CFs to muscle-like cells, allowing it to be used as a bridge to heart transplantation or as an ideal peptide drug for cardiac regeneration therapy.

Conflict of interest: none declared.

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