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# Wnt/frizzled signalling modulates the migration and differentiation of immortalized cardiac fibroblasts

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**Aims** The Wnt/frizzled (Fzd) signal transduction cascade has been implicated in the proliferation, differentiation, and migration of many cell types, but the role of this pathway in cardiac fibroblast differentiation is not known. Our lab previously showed an up-regulation of Fzd-1 and -2 expression in myofibroblasts after myocardial infarction (MI), indicating a potential role for the Fzd receptor in fibroblast–myofibroblast differentiation. The present study was performed to further define the role of specific Wnt and Fzd proteins in the proliferation, migration, and differentiation of cardiac fibroblasts.

**Methods and results** Because primary fibroblasts become senescent after a few passages and are difficult to transfect, we immortalized rat cardiac fibroblasts with telomerase [cardiac fibroblasts immortalized with telomerase (CFIT)]. Proliferation of CFIT was not significantly influenced by Wnt/Fzd signalling. The migration, however, was attenuated by all Wnt/Fzd combinations tested. Also, specific Wnt/Fzd combinations modulated the expression of the following myofibroblast markers: collagen  $\alpha 1$ , collagen III, fibronectin and its splice variants, and  $\alpha$ -smooth muscle actin.

**Conclusion** The results indicate that myofibroblast migration and differentiation, but not proliferation, can be modulated by interventions in Wnt/Fzd signalling. Therefore, Wnt/Fzd signalling may serve as a novel therapeutic target to ameliorate wound healing after MI.

**Keywords** Myofibroblast • Heart • Wnt/frizzled signalling • Differentiation • Migration

## 1. Introduction

Myocardial infarction (MI) is one of the main causes of mortality and morbidity in Western countries. Many patients survive the acute phase after MI as a result of improved treatment. Therefore, more patients enter the wound healing phase after MI. In this process, the dead cardiomyocytes are replaced by granulation tissue, which eventually matures into a scar.<sup>1,2</sup> Inadequate wound healing will lead to dilatation of the heart and loss of cardiac function, resulting in heart failure.<sup>2,3</sup> Because current pharmacological therapies slow down the progressive deterioration of cardiac function but cannot reverse this process, the only rational therapy for heart failure is to prevent its development.<sup>4,5</sup>

Myofibroblasts play an important role in wound healing in many organs including the heart.<sup>6</sup> These cells migrate into the infarct area

in the second week after MI, where they form granulation tissue. Their contractile properties, combined with their synthesis of extracellular matrix proteins, are thought to prevent infarct expansion.<sup>7</sup> The sustained presence and ongoing activity of myofibroblasts in the scar tissue in a well-healed infarct suggests an important role of these cells in the maintenance of the extracellular matrix that has been deposited in the infarct area.<sup>8–10</sup>

The process of differentiation and migration of (myo)fibroblasts in the infarct area is far from completely elucidated. Next to a role for TGF- $\beta$  and mechanical stretch,<sup>6</sup> accumulating evidence suggests a role for Wnt/frizzled (Fzd) signalling in this context. During their migration into the infarct area, Fzd-1 and -2 receptors and the intracellular signal transduction molecule dishevelled-1 (Dvl-1) are expressed in the myofibroblast area.<sup>11,12</sup> Moreover, overexpression of soluble

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Fzd-related protein or the second messenger  $\beta$ -catenin has been shown to reduce infarct expansion and improves cardiac function after MI.<sup>13–16</sup> Wnts are highly conserved secreted glycoproteins, with an important function during development and disease.<sup>17,18</sup> Fzds are the predominant receptors for these Wnt ligands, with a seven transmembrane structure.<sup>19</sup> The Wnt/Fzd signalling is very complex and can be subdivided into  $\beta$ -catenin-dependent and -independent pathways.<sup>20</sup> Calcium serves as a second messenger in the latter pathway.<sup>21–24</sup>

The aim of the present study was to further elucidate the role of Wnt/Fzd signalling in the proliferation, differentiation, and migration of cardiac (myo)fibroblasts *in vitro*. So far, this research was hampered by three adverse characteristics of primary cardiac fibroblasts in culture: (i) spontaneous differentiation to myofibroblast phenotype when cultured on rigid culture dishes;<sup>25</sup> (ii) poor transfection efficiency;<sup>26</sup> and (iii) senescence after approximately five passages.<sup>27,28</sup> To overcome these problems, we have developed an immortalized cardiac fibroblast cell line by stably overexpressing telomerase in these cells.<sup>29</sup> This cell line which we named CFIT (cardiac fibroblasts immortalized with telomerase) exhibits a fibroblast phenotype even after multiple passages and shows high transfection efficiency. These characteristics make this cell line an excellent tool to study the effects of Wnt/Fzd signalling on cardiac fibroblast proliferation, migration, and differentiation.

## 2. Methods

### 2.1 Materials

The following chemicals were used: ( $\pm$ )-verapamil hydrochloride, a calcium antagonist (Sigma-Aldrich, Saint Louis, MO, USA), and BayK8644 (Bayer, Mijdrecht, The Netherlands), a calcium agonist. For the proliferation assay, 5-bromo-2'-deoxyuridine (BrdU) and BrdU antibody, diaminobenzidine tetrahydrochloride (DAB), as well as the secondary antibody rabbit-anti-mouse biotin, were used (Sigma-Aldrich). siRNAs directed against Fzd-1 and -2 are all pools obtained from Dharmacon, Chicago, IL, USA. siRNA directed against GFP was used as a negative control. Recombinant Wnt3a (rWnt3a) was obtained from R&D systems, Abingdon, UK.

### 2.2 Cell culture and transient transfection

The development of the CFIT cell line has been described elsewhere.<sup>29</sup> Briefly, cardiac fibroblasts were isolated from male Lewis rats, as described previously<sup>30</sup> and transfected with the pGRN145 plasmid<sup>31</sup> containing human telomerase reverse transcriptase (ATCC, Middlesex, UK). These experiments were performed under conditions stated in 'the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996)' and approved by the Universities Ethics Review Board of Maastricht University.

CFITs were cultured in 75 cm<sup>2</sup> culture flasks (Costar Corning, Schiphol, The Netherlands) in Dulbecco's modified essential medium with L-glutamine (2 mM), 10% foetal calf serum (Invitrogen, Merelbeke, Belgium), and 50 mg/mL gentamycin (Sigma-Aldrich). Before starting the experiment, the cell line was treated with plasmocin 25  $\mu$ g/mL (Invivogen, Toulouse, France) and tested with a mycoalert mycoplasma detection kit (Lonza, Rockland, ME, USA). CFITs were transiently transfected with pcDNA3.1/hygro (Invitrogen) containing either Wnt3a, rFzd-1, or -2, with pcDNA3.0 (Invitrogen) containing  $\beta$ -catenin or hFzd-5 or with siRNA. Transient transfections were performed with Fugene6 (Roche, Indianapolis, IN, USA) and 1  $\mu$ g/mL plasmid DNA. After transfection, the cells were cultured for two more days, before the experiments

started. Conditioned medium was collected from the L-cells that stably overexpress either Wnt3a or Wnt5a (Invitrogen), as described previously.<sup>32,33</sup> Conditioned medium from unmanipulated L-cells was used as a control. A TOPFlash luciferase assay (see Section 2.9) was used to calculate the amount of Wnt in the conditioned medium. We constructed a calibration curve generated with purified rWnt3a protein and compared the luciferase activity of our conditioned medium with it. This procedure was repeated for all new batches to guarantee the use of equal amounts of Wnt3a protein in all experiments. For all experiments, a final Wnt concentration of  $1.10^{-8}$  M was used.

### 2.3 Proliferation assay

On the first day, cells were plated and the next day, at 70% confluence, these cells were transfected or treated. Proliferation rates were measured after 48 h with a BrdU incorporation assay. Cells were incubated with 100  $\mu$ M BrdU for 1 h and afterwards fixed with methanol/acetone (1:1) for 20 min at 4°C. After washing three times with PBS, cells were incubated in 2 N HCl for 20 min at 37°C, washed three times with water, and incubated in 0.1 M sodium borate (pH 8.5) for 10 min at room temperature. After washing in PBS, the cells were incubated with primary monoclonal anti-BrdU antibody (1:1000 in PBS/1%BSA/0.1%Tween 20, Sigma-Aldrich) for 30 min at 37°C, washed three times with PBS, and incubated with biotinylated rabbit-anti-mouse antibody (1:400, DAKO) for 30 min at room temperature. After washing five times with PBS, the cells were incubated in ABC-AP solution and the labelled products were visualized using 5 mg/mL DAB, 10% of 0.1 M imidazole, 50  $\mu$ L of 30% hydrogen peroxide in 100 mL of 0.05 M Tris/HCl buffer (pH 7.6).

### 2.4 Wound assay

Cells were plated on day 0 and cultured until 70% confluence before transfection or treatment (see above). The following substances were used: ( $\pm$ )-verapamil hydrochloride (1  $\mu$ M) and BayK8644 (10  $\mu$ M). Migration assays started 48 h after transfection and/or treatment by scratching the monolayer with a pipette tip (1 mm width). The time point where the scratch was made was taken as 0 h. Scratch width was measured at this time point and after 6, 12, and 24 h. For all the results, the scratch at 0 h was set as 100% wound width and further results were expressed relative to this value.

### 2.5 Differentiation assay

The same timelines and protocols were used as described above. RNA was isolated using the Trizol method (Invitrogen) and cDNA prepared with the iscript<sup>TM</sup> cDNA kit (Bio-Rad, Hercules, CA, USA). qPCR was performed as described previously.<sup>34</sup> The following myofibroblast markers were determined:  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA),  $\alpha$ 1 type I collagen (Col1 $\alpha$ 1), type III collagen, total fibronectin (FN), and its EDA and EDB splice variants (EDA-FN and EDB-FN). Cyclophilin served as the house-keeping gene. Further, qPCRs were performed on all samples, with a TGF- $\beta$ , Fzd-1 and -2, and Wnt3a and Wnt5a primer set (for primer sequences, see Supplementary material online, Table S1).

For western blot, cells were harvested with trypsin, pelleted and placed in 500  $\mu$ L ice-cold Laemmli buffer (6.6% glycerol, 1.5% SDS, and 4.15 mmol/L Tris/HCl, pH 8.0), and homogenized. After 30 min on ice and centrifugation, the supernatants were collected and protein content was measured using the BCA protein assay (Pierce Biotechnology Inc., Rockford, IL, USA); 10  $\mu$ g of total protein was denatured by boiling in Laemmli sample buffer (Bio-Rad), separated on a 10% SDS-PAGE, and transferred onto a Hybond C nitrocellulose membrane (GE Healthcare, Little Chalfont, UK). After blocking [5% non-fat dry milk (Bio-Rad) and 0.1% Tween in TBS] for 1 h, membranes were incubated overnight at 4°C with primary antibodies directed against  $\beta$ -catenin 1/2000 (BD Biosciences, Franklin Lakes, NJ, USA),  $\alpha$ -SMA, total collagen, and  $\beta$ -actin 1/2000 (Sigma-Aldrich). Anti-rabbit or -mouse immunoglobulin G 1/5000

(Vector Laboratories, Burlingame CA, USA) was used as the secondary antibody, and the membranes were developed using the Supersignal West Pico chemiluminescence kit (Pierce). Images of the blots were analysed with image analysis software (Leica QWin, Cambridge, UK).

## 2.6 Immunofluorescence

The same timelines and protocols were used as described above. Cells were washed with PBS and fixed with methanol/acetone (1:1). After incubation for 5 min with 0.5% Triton, cells were washed with PBS/glycine and incubated with 2% BSA. Overnight, the cells were incubated with the primary  $\alpha$ -SMA antibody (Sigma-Aldrich) and 2 h with secondary Alexa fluor 488 goat anti-mouse antibody (Vector Laboratories). Cell nuclei were visualized by vectashield mounting medium with DAPI (Vector Laboratories). Photos were taken with a Leica CTR500 camera and analysed with the Quantimet program (Leica QWin/QGo).

## 2.7 Determination of collagen excretion

Cells were pre-treated as described above. Two hundred and fifty microlitres of cell suspension was mixed with 1.25 mL colour reagent (250 mg sirius red solved in 250 mL picric acid with 1.25 mL Triton X-100). The solution was centrifuged (3 min at 5.000 g) and the colour reagent was discarded. The pellet was resuspended in 0.3 mL of 0.5 M NaOH, and this solution was incubated for 10 min and measured with an extinction of 540 nm.

## 2.8 Collagen gel contraction

Collagen gel contraction experiments were performed according to the method described by Lijnen et al.<sup>35,36</sup> The wells were coated with 1% BSA for 1 h at 37°C. In the mean time, CFITs were harvested and resuspended in a final volume of  $1 \times 10^6$  cells/mL. Prior to this experiment, cells were pre-treated as described above. The BSA was aspirated from the plate and the collagen mix [50% of 3 mg/ml collagen I (Invitrogen) and 50% of  $2 \times$  DMEM] was added to the cells immediately before plating. Gels were made floating by nudging with a pipette tip and placed at 37°C for 24 h, before the gels were assessed. The gel diameter was used as a measure of myofibroblast contraction.

## 2.9 Calcium and luciferase assay

Calcium measurements were performed at different time points after treatment or transfection, using the Fluo-4NW calcium assay kit (Invitrogen). For the luciferase experiments, the cells were additionally transfected with a TOPFlash construct consisting of 8TCF/LEF-binding sites cloned stably into the Mlu1 site of a pTA-Luc vector. The construct was kindly provided by Dr J. Nathans, Baltimore MD, USA. Luciferase activity was measured using a luciferase assay system (Promega, Madison WI, USA).

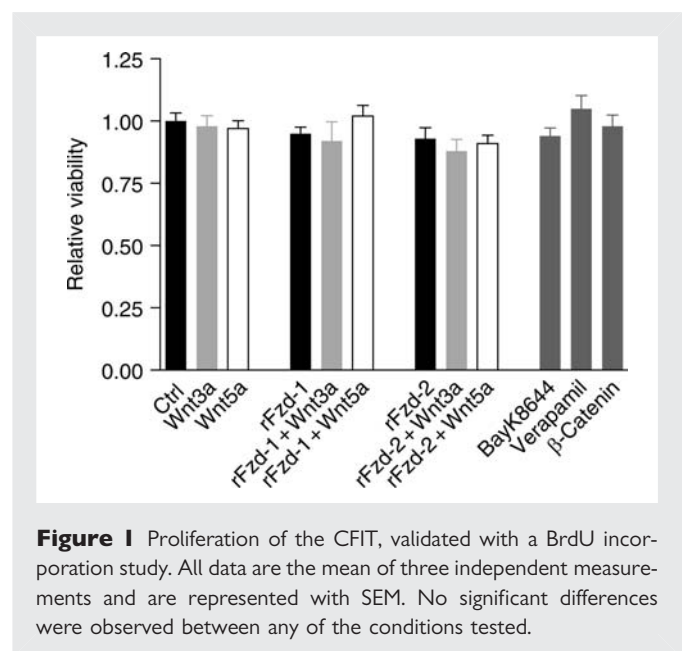
## 2.10 Statistical analysis

All values are shown as mean  $\pm$  SEM. Differences between groups were examined for statistical significance using two-way ANOVA with the Bonferroni *post hoc* test. A *P*-value  $< 0.05$  was considered to indicate a statistically significant difference.

# 3. Results

## 3.1 Effect of Wnt/Fzd signalling on CFIT proliferation

Cells were exposed to either Wnt3a or Wnt5a conditioned culture medium ( $1.10^{-8}$  M) either alone or in combination with rFzd-1 or -2 transfection. All experiments were repeated with purified rWnt and the Wnt constructs to exclude non-specific effects of the conditioned medium. The contribution of downstream signalling

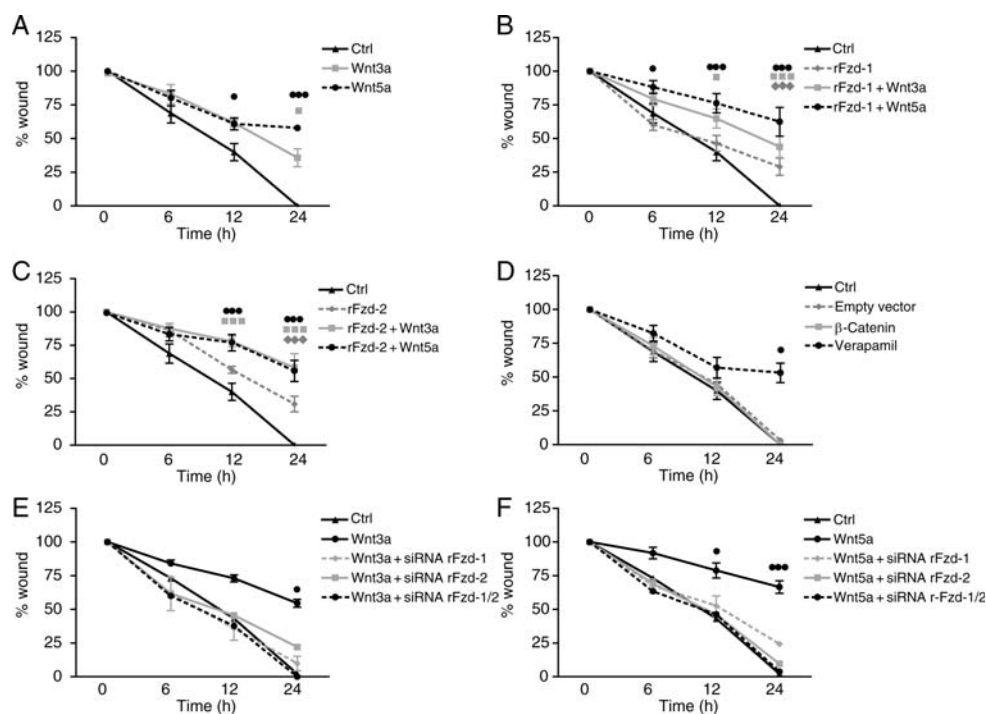


components was investigated by overexpression of  $\beta$ -catenin or by administration of the calcium agonist BayK8644 or the calcium antagonist ( $\pm$ )-verapamil. As shown in Figure 1, neither of the combinations of Wnts and Fzds induced a significant change in the proliferation rate of CFIT compared with the unmanipulated control. No effect on CFIT proliferation was observed by interventions in the downstream signalling.

## 3.2 Effect of Wnt/Fzd signalling on CFIT migration

Migration of CFIT was determined using an *in vitro* wound assay, in which the closure of a scratch in the cell layer was followed in time. In Figure 2A, the attenuation of wound closure by Wnt3a or Wnt5a is shown. Transfection of either rFzd-1 (Figure 2B) or rFzd-2 (Figure 2C) also induced the attenuation of the wound closure of CFIT, which was further enhanced by the addition of Wnt3a or Wnt5a. Administration of rWnts or transient Wnt transfection yielded similar results (data not shown). In Figure 2D, we further investigated the downstream signalling pathways involved in the anti-migratory effect of Wnt/Fzd signalling. Overexpression of  $\beta$ -catenin, the second messenger of  $\beta$ -catenin-dependent Wnt signalling pathway, did not affect the migration of CFIT. In contrast, the calcium antagonist ( $\pm$ )-verapamil attenuated the migration to a similar extent as the interventions in Wnt/Fzd signalling. These results suggest that calcium-mediated Fzd signalling, rather than  $\beta$ -catenin-mediated signalling, is responsible for the inhibitory effect on CFIT migration.

To assess the contribution of the endogenous Fzd receptors to the effects of Wnt3a and Wnt5a, siRNAs for rFzd-1 and -2 were transfected into CFIT (Figure 2E and F). Transfection of either of the siRNAs reduced the effect of Wnt3a or Wnt5a administration, whereas combined transfection of the siRNAs completely abolished the effect of Wnt3a and Wnt5a on CFIT migration. These experiments suggest that both endogenous rFzd-1 and -2 receptors are involved in the effects of Wnt3a and Wnt5a on CFIT migration. Because high concentrations of siRNAs could inhibit protein synthesis



**Figure 2** Migration of CFIT into a scratch in a confluent cell layer. (A) Wnt3a and Wnt5a induced a significant delay of migration at 12 h compared with control, which was even more pronounced at 24 h. (B) Effects of rFzd-1, either alone or in combination with Wnt3a or Wnt5a, on CFIT migration. rFzd-1 by itself delayed CFIT migration, and the effect was even more pronounced when combined with Wnt3a or Wnt5a ( $n = 5$ ). (C) A similar pattern of inhibition was observed for rFzd-2 ( $n = 5$ ). (D) Empty vector and  $\beta$ -catenin had no influence on CFIT migration. ( $\pm$ )-Verapamil, on the other hand, delayed the migration significantly. (E) Effect of inhibition of the rFzd-1 and -2 expression on the anti-migratory effects of Wnt3a. The combination of the two siRNAs completely prevented the effects of Wnt3a on the delay of CFIT migration. (F) For Wnt5a, siRNAs exhibited the same effect as shown for Wnt3a. All data are expressed as mean with SEM (\* $P < 0.05$  and \*\*\* $P < 0.001$ ).

or lead to non-specific reactions, we also tested an siRNA directed against GFP. This siRNA did not affect the migration of CFIT (data not shown).

To find out whether the delayed wound closure is a consequence of a migratory effect or a combination of delayed migration and decreased proliferation at the site of injury, CFITs were exposed to BrdU during the migration. No significant differences in proliferation rate could be observed (data not shown). This indicated that the delayed CFIT wound closure is the sole consequence of a migratory effect.

### 3.3 Effect of Wnt/Fzd signalling on CFIT differentiation

To determine the effect of activation of Wnt/Fzd signalling on fibroblast-to-myofibroblast differentiation of CFIT, we determined the expression levels of different myofibroblast markers including  $\alpha$ -SMA, Collagen Ia1 and III, FN, and its splice variant EDA-FN and EDB-FN using qPCR (Figure 3A–F). Administration of Wnt3a to the culture medium significantly reduced the expression of all differentiation markers except EDB-FN, whereas administration of Wnt5a had no effect. Overexpression of either rFzd-1 or -2 alone did not affect the expression of any of the differentiation markers.

Combination of Fzd overexpression and Wnt administration revealed opposite effects for rFzd-1 and -2. In CFIT cells overexpressing rFzd-1, Wnt3a addition stimulated the expression of myofibroblast markers, whereas Wnt5a administration showed an inhibitory

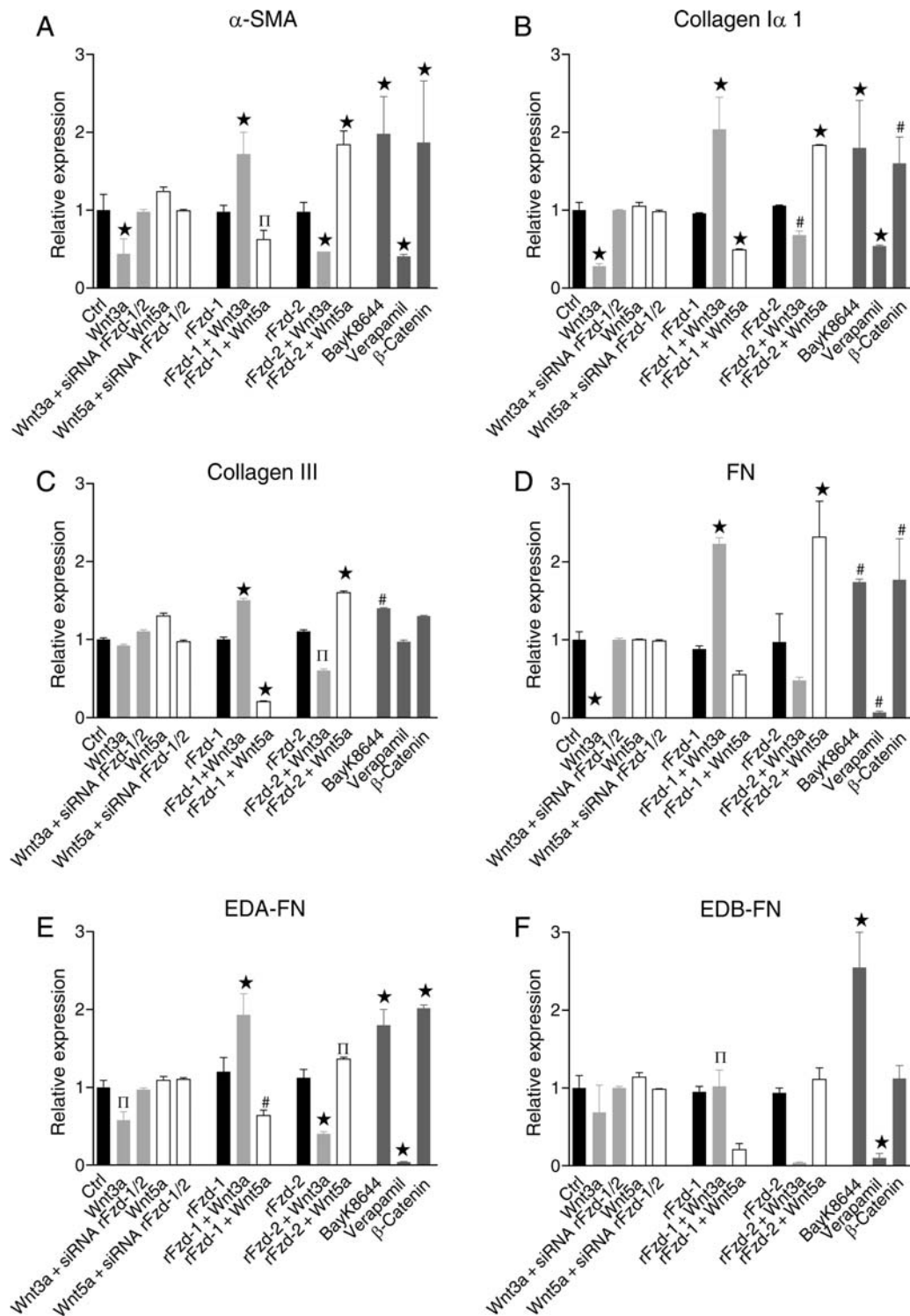
effect. In contrast, in CFIT cells overexpressing rFzd-2, Wnt3a inhibited the expression of myofibroblast markers, whereas Wnt5a showed a stimulatory effect. All these experiments were repeated with rWnt protein and with transient transfection of the Wnt constructs, yielding similar results as obtained with conditioned culture medium. A direct activation of  $\beta$ -catenin-mediated Fzd signalling by  $\beta$ -catenin overexpression induced myofibroblast differentiation. A similar result was obtained by activation of  $\text{Ca}^{2+}$  signalling by BayK8644, whereas the  $\text{Ca}^{2+}$  antagonist ( $\pm$ )-verapamil induced a fibroblast phenotype with low levels of  $\alpha$ -SMA.

### 3.4 Analysis of the differentiation at the protein level

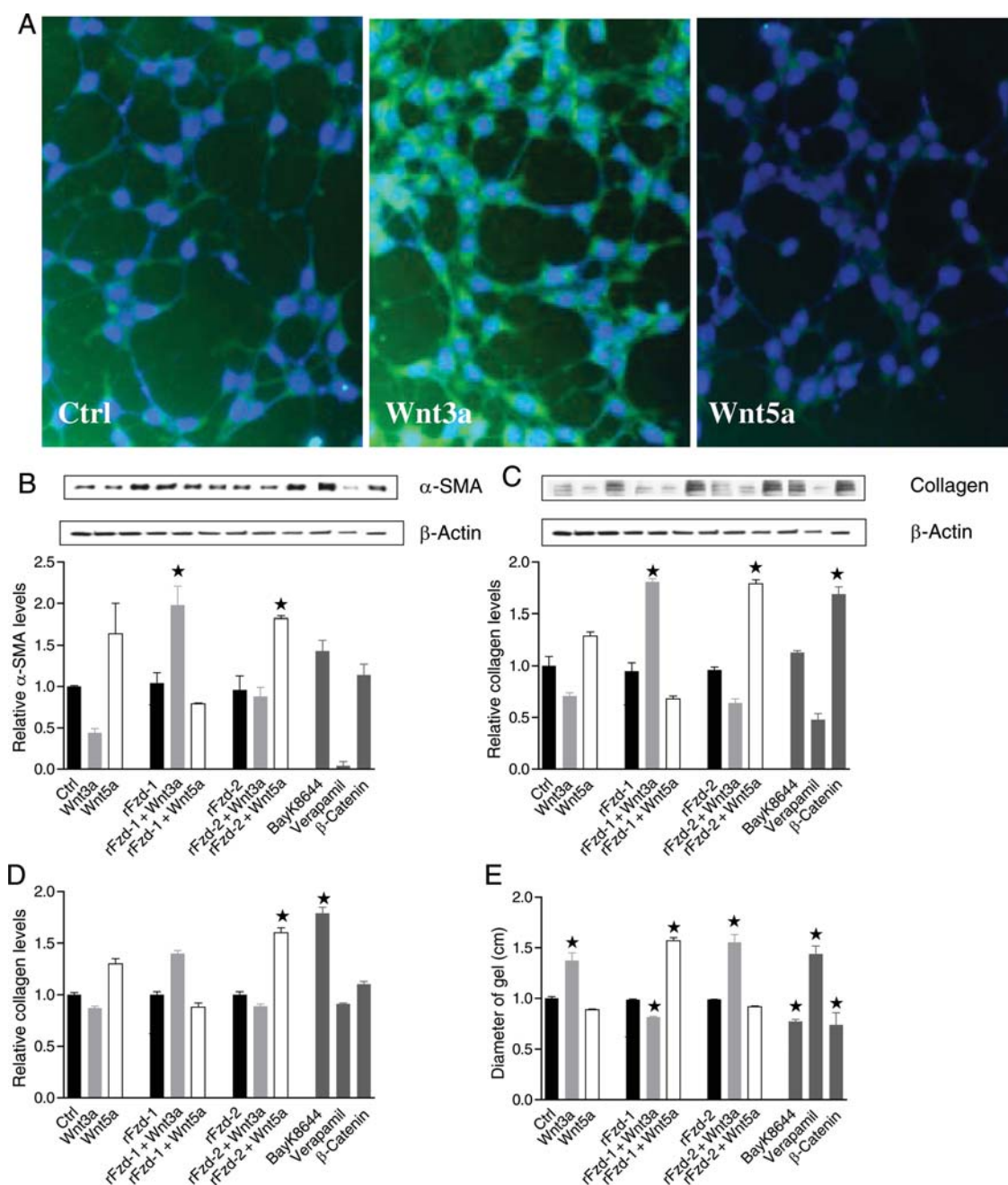
Immunocytochemistry for  $\alpha$ -SMA was performed on CFIT cell cultures stimulated with Wnt3a or Wnt5a. As shown in Figure 4A, the addition of Wnt5a induced the expression of  $\alpha$ -SMA, whereas Wnt3a treatment did not. In Figure 4B, protein levels of  $\alpha$ -SMA as determined by western blotting are presented. A combination of transfection of rFzd-1 and Wnt3a, and of transfection of rFzd-2 and Wnt5a, induced  $\alpha$ -SMA expression. These data confirm the pattern observed for the  $\alpha$ -SMA expression at the mRNA level (Figure 3A).

Furthermore, the protein levels of total collagen were determined by western blotting (Figure 4C), confirming the differentiation patterns as presented in Figures 3A, and 4A and B. A similar pattern of collagen levels could be observed in the CFIT culture medium (Figure 4D).

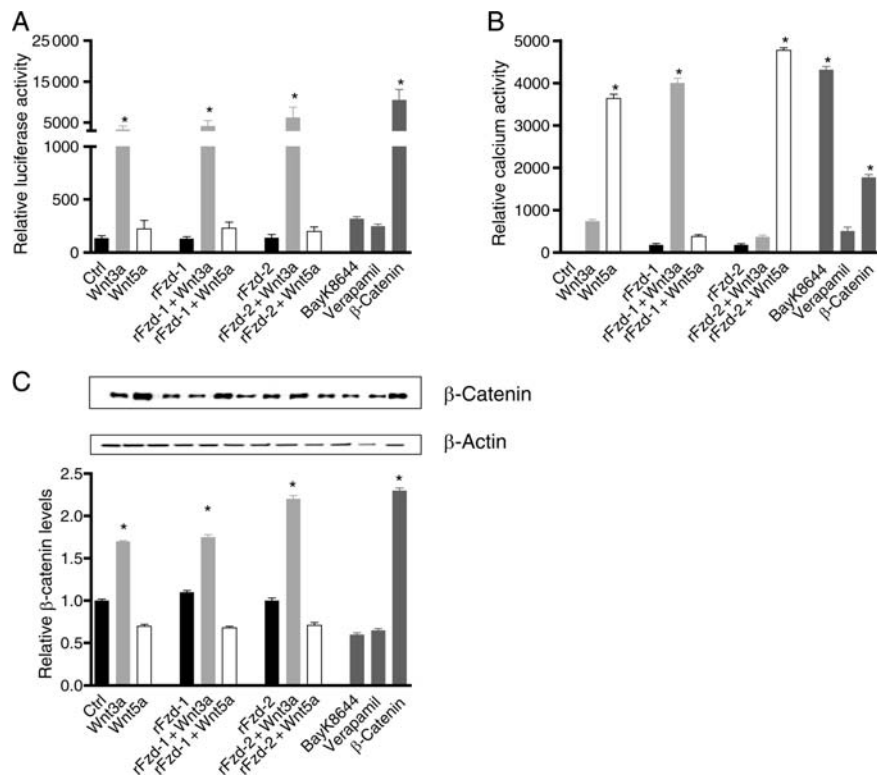




**Figure 3** Effect of Wnt/Fzd signalling on the expression of myofibroblast differentiation markers in CFIT, determined by qPCR. Expression levels are corrected for cyclophilin expression and normalized to the control values. The results are the mean of ten different measurements of five different transfection rounds ( $^{\#}P < 0.05$ ,  $^{\text{II}}P < 0.01$ , and  $^{\text{I}^*}P < 0.001$ ). The different differentiation markers are (A)  $\alpha$ -SMA, (B) Col I $\alpha$ 1, (C) collagen III, (D) FN, (E) EDA-FN, and (F) EDB-FN. Overexpression of the Fzd receptors alone had no effect. In combination with Wnt ligands, we observed ligand/receptor specificity for the differentiation. Overexpression of  $\beta$ -catenin and administration of BayK8644 stimulated the expression of myofibroblast differentiation markers, whereas ( $\pm$ )-verapamil potentially inhibited the expression of most of the differentiation markers.



**Figure 4** Effects of Wnt administration on the differentiation of CFIT cells on protein level. (A) Immunocytochemistry for  $\alpha$ -SMA is shown in the absence and presence of either Wnt3a or Wnt5a. It is clear that the total amount of  $\alpha$ -SMA is increased after Wnt3a conditioned medium and decreased after treatment with Wnt5a conditioned medium, compared with control. (B) A western blot for  $\alpha$ -SMA and (C) total collagen was performed. The effects of different Wnt/Fzd combinations on  $\alpha$ -SMA protein were similar to those observed on mRNA level (Figure 3A–C). (D) Relative collagen content in the medium of CFIT exposed to different Wnt/Fzd combinations. These data confirm the western blot data, in which intracellular collagen content was determined. All results shown are means with SEM and were obtained from three independent measurements of six different transfections ( $^*P < 0.001$ ). (E) A collagen gel assay was used to assess the contractile properties of CFIT under different conditions. Gels were measured after 24 h and their dimensions related to gels were cultured under control conditions. All results shown are means with SEM and were obtained from two independent measurements of four different transfections.



**Figure 5** Assessment of different intracellular signalling pathways activated in CFIT cells by different Wnt/Fzd combinations. (A) TOPFlash assays confirm that Wnt3a could activate  $\beta$ -catenin-mediated Wnt signalling, whereas Wnt5a could not. (B) The  $\beta$ -catenin-independent signalling was studied using a calcium assay. Intracellular calcium levels exhibited the same pattern as shown for the differentiation, indicating that the  $\beta$ -catenin-independent pathway is the main controller of CFIT differentiation. (C) To confirm that differentiation is not controlled by  $\beta$ -catenin, we determined the content of this protein by western blot. These data were in agreement with results from the TOPFlash assays. All results shown are means with SEM and were obtained from three independent measurements of six different transfections (\* $P < 0.001$ ).

To correlate the increased expression levels of myofibroblast markers with a more contractile phenotype of the CFIT, collagen contraction assays were performed. CFIT were transfected and cultured for 48 h, detached from their culture dish and added to a collagen gel. These experiments showed that elevated  $\alpha$ -SMA protein levels led to a decreased gel size, whereas reduced  $\alpha$ -SMA protein levels led to increased gel size, when compared with unmanipulated controls (Figure 4E).

### 3.5 Activation of different intracellular signal transduction cascades

To elucidate the opposing effects of Wnt3a and Wnt5a on the differentiation of CFIT, the activation of the two main signalling transduction cascades was studied.  $\beta$ -Catenin-mediated signalling was assessed by determining the activity of luciferase produced by a TCF–LEF-luciferase construct (TOPFlash assay). The addition of Wnt3a increased luciferase activity, whereas Wnt5a did not. These results confirm the literature where Wnt3a-mediated signalling is considered to be  $\beta$ -catenin-dependent and Wnt5a signalling is  $\beta$ -catenin-independent (Figure 5A). However, this activation pattern showed no correlation with the differentiation pattern. Therefore, intracellular calcium levels were determined as a measure of  $\beta$ -catenin-independent Wnt signalling (Figure 5B). Calcium levels clearly correlated with CFIT differentiation, indicating that the

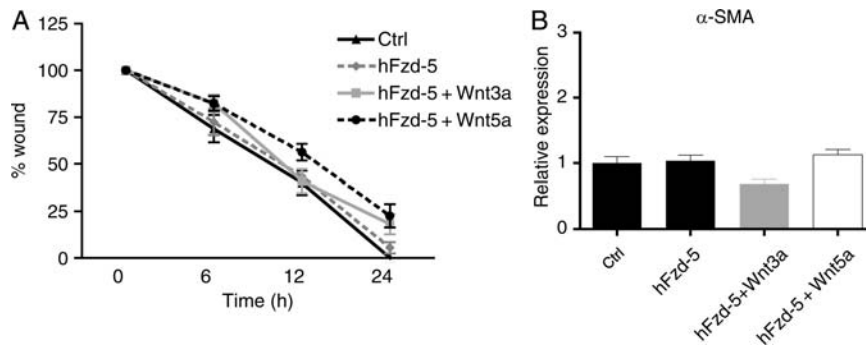
second messenger calcium was the main controller of the differentiation of these cells. To confirm that the second messenger of the  $\beta$ -catenin-independent pathway had no direct influence on the  $\beta$ -catenin-dependent signalling,  $\beta$ -catenin protein levels were examined by western blot. These data confirm that the TOPFlash assay was an accurate functional assessment of  $\beta$ -catenin-dependent activity and that the  $\beta$ -catenin-dependent pathway was not the main pathway in the migration and differentiation (Figure 5C).

Next, we studied a possible interaction between Wnt signalling and TGF- $\beta$ , a growth factor known to influence the differentiation of fibroblast to myofibroblast. Manipulation of neither  $\beta$ -catenin-dependent or -independent Wnt signalling affected the mRNA levels of TGF- $\beta$ . This indicated that the Wnt/Fzd pathway is independent of the TGF- $\beta$  pathway (data not shown).

### 3.6 Specificity of the Fzd receptors involved in CFIT migration and differentiation

To study the selectivity of the observed effects, we subsequently tested the effect of overexpression of hFzd-5 on CFIT migration. Overexpression of hFzd-5 alone did not attenuate the migration, whereas the attenuating effect of Wnt3a or Wnt5a on CFIT migration, shown in Figure 2, was not modified by the overexpression of hFzd-5 (Figure 6A). Overexpression of hFzd-5 also did not alter the





**Figure 6** (A) Transfection of hFzd-5 had little effect on CFIT migration. All data are a mean of three independent experiments. (B) Differentiation of CFIT was not affected by hFzd-5 overexpression. These experiments are a combination of three different transfections and a total of six samples ( $n = 6$ ). The error bars represent SEM.

expression of  $\alpha$ -SMA on both protein (data not shown) and mRNA level (Figure 6B), either alone or in combination with Wnt3a or Wnt5a. This indicates that hFzd-5 is not capable of transducing the Wnt signals in CFIT.

## 4. Discussion

A significant fraction of MI patients develops heart failure, a condition that is due to the development of excessive dilatation of the infarct area, the so-called infarct expansion.<sup>37,38</sup> It is therefore of vital importance to better understand the wound healing process after MI and to identify new therapeutic targets that can help to improve this process.<sup>1,3</sup> We and others have demonstrated the crucial role of the myofibroblast in the prevention of cardiac dilatation after MI.<sup>6,7,39,40</sup> So far, the signalling pathways involved in myofibroblast differentiation are not fully elucidated.

In the infarcted heart, myofibroblasts can be observed in the infarct area but also in areas remote from the ischaemic zone.<sup>40</sup> In the infarct area, these cells serve a dual role: they contract similar to smooth muscle cells and subsequently deposit extracellular matrix proteins. These actions result in a smaller and stronger scar area that helps to prevent infarct expansion and ventricular dilatation. The presence of myofibroblasts in the infarct area is important to ensure maintenance of the extracellular matrix which is subjected to wear and tear in the beating heart.<sup>37</sup> However, excessive matrix deposition in remote areas contributes to stiffening of the ventricular wall and adverse remodelling, leading to heart failure.<sup>41</sup> The involvement of local angiotensin production in the fibrotic response has been firmly established<sup>42</sup> and inhibitors of the renin-angiotensin system are capable of limiting this remote fibrosis.<sup>43,44</sup> However, these drugs cannot prevent heart failure development in a significant fraction of the MI patients and have been reported to have pleiotropic effects.<sup>5</sup> This underscores the importance of identifying novel therapeutic targets for adverse cardiac remodelling.

One of the aims of the present study was to elucidate the role of Wnt/Fzd signalling in fibroblast-to-myofibroblast differentiation. After an initial report by our group describing the overexpression of rFzd-2 and Dvl-1 in myofibroblasts in the healing rat infarct,<sup>11,45</sup> Barandon *et al.*<sup>13</sup> demonstrated that overexpression of FrzA, a member of the soluble Fzd-related protein family, improved infarct healing in mice.

This family of proteins is thought to interfere with Wnt/Fzd signalling by scavenging Wnt proteins, although alternative signalling routes have been suggested.<sup>46</sup> Subsequently, this observation has been confirmed by other groups.<sup>12,14,15</sup> In addressing the aim of the present study, we realized that *in vivo* studies are not well suited to sort out the underlying molecular mechanisms of wound healing, since many cell types and signalling pathways are active at the same time.

To this end, we developed a cardiac fibroblast cell line that is immortalized with telomerase.<sup>29</sup> In contrast to primary cultures of cardiac fibroblasts, this cell line shows little spontaneous differentiation into myofibroblasts, making it a suitable tool to study the effect of Wnt/Fzd signalling on myofibroblast differentiation. We observed that overexpression of rFzd-1 or -2, either alone or in combination with Wnt3a or Wnt5a administration, had no effect on CFIT proliferation. In contrast, CFIT migration was clearly attenuated by either rFzd-1 or -2 overexpression, and this effect could be enhanced by additional administration of Wnt3a or Wnt5a to the cells. The effect could be mimicked by interventions in the  $\text{Ca}^{2+}$  metabolism, but overexpression of  $\beta$ -catenin had no effect on CFIT migration. This indicates that non- $\beta$ -catenin-mediated rather than  $\beta$ -catenin-mediated Wnt/Fzd signalling is responsible for the attenuation of CFIT migration. We also observed attenuated CFIT migration when Wnt3a or Wnt5a was administered alone, without overexpression of Fzd receptors. Blocking of rFzd-1 and -2 expression by siRNA transfection showed that this effect could be attributed to the activation of endogenous rFzd-1 and -2 receptors.

Migration of (myo)fibroblasts into the areas of myocyte loss is an important step in the process of wound healing. Chemotactic factors released by inflammatory cells stimulate the migration of these cells into the infarct area.<sup>47</sup> Mechanisms that control the process of migration, however, are largely unknown. The results of the present study show that activation of Wnt/Fzd signalling attenuates the CFIT migration. From these data, we conclude that fibrosis can be controlled not only at the level of fibroblast proliferation and differentiation into myofibroblasts, but also at the level of migration of these cells into the injured areas of the heart.

Interventions in Wnt/Fzd signalling also modulated the differentiation of CFIT into a myofibroblast phenotype. However, opposite effects were observed for different combinations of Wnts and Fzds: differentiation was stimulated with the combinations rFzd-1/Wnt3a

and rFzd-2/Wnt5a, whereas the combinations rFzd-1/Wnt5a and rFzd-2/Wnt3a exerted an anti-differentiating effect. This underlines the complexity of the Wnt/Fzd signalling in the induction of myofibroblast differentiation. Interventions in  $Ca^{2+}$  metabolism were shown to affect CFIT differentiation, whereas overexpression of  $\beta$ -catenin had no effect. Therefore, our data suggest that the  $\beta$ -catenin independent signalling is the main pathway for the effects of Wnt on the differentiation of CFIT. One has to conclude that our current knowledge of the pharmacology of the Wnt/Fzd pathway and their preferential activation of either of the different signal transduction pathways induced by different combinations of Wnts and Fzds is far from complete,<sup>48</sup> which sometimes can lead to seemingly contradictory results. However, our data increase the knowledge of the effect of  $\beta$ -catenin-independent signalling on fibroblast migration and differentiation.

Recently, telomerase has been shown to modulate Wnt signalling in both *in vivo* and in embryonic stem cells by interacting with  $\beta$ -catenin target gene transcription.<sup>49</sup> This mechanism, however, is not likely to influence the outcome of the experiments reported here, since all experimental groups in the present study were derived from identical batches of CFIT. Moreover, the effects of Wnt signalling on CFIT migration could be attributed to non- $\beta$ -catenin-mediated Wnt signalling, whereas the modulation of differentiation resulted from both  $\beta$ -catenin- and non- $\beta$ -catenin-mediated Wnt signalling.

In conclusion, interventions at the level of Wnt/Fzd signalling have an effect on the migration and the differentiation of cardiac fibroblasts. These results reported here combined with the *in vivo* data presented above underscore that appropriate interventions in Wnt/Fzd signalling at the level of the (myo)fibroblast can serve as a novel therapeutic approach to prevent excessive dilatation of the infarct area and preserve cardiac function after MI.

## Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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