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# Dissecting the impact of Frizzled receptors in Wnt/ $\beta$ -catenin signaling of human mesenchymal stem cells

**Abstract:** Wnt/ $\beta$ -catenin signaling is of fundamental importance in the regulation of self-renewal, migration/invasion, and differentiation of human mesenchymal stem cells (hMSCs). Because little information is available about the function of Frizzled receptors (Fzds) as the main receptors of Wnt proteins in hMSCs, we first performed comparative Fzd mRNA expression profiling. Fzd9 and Fzd10 were not expressed in hMSCs. While Fzd3 was expressed at low levels in hMSCs, the other Fzds exhibited high expression rates. Activation and repression of Wnt signaling in hMSCs revealed that the expression levels of Fzd1, Fzd6, and Fzd7 are positively correlated with the Wnt/ $\beta$ -catenin activation status, whereas Fzd8 exhibited an inverse relation. For studying the functional relevance of Fzds in Wnt/ $\beta$ -catenin signaling, RNA interference, ectopic expression studies, and rescue approaches were performed in hMSCs carrying a highly sensitive TCF/LEF reporter gene system (*Gaussia* luciferase). We found that, Fzd1, Fzd5, Fzd7, and Fzd8 are largely involved in Wnt/ $\beta$ -catenin signaling of hMSCs. Moreover, the knockdown of Fzd5 can be compensated by the ectopic expression of Fzd7. Conversely, the ectopic expression of Fzd5 in Fzd7-knockdown hMSCs resulted in a rescue of Wnt/ $\beta$ -catenin signaling, pointing to a functional redundancy of Fzd5 and Fzd7.

**Keywords:** Frizzled receptors (Fzds); human mesenchymal stem cells (hMSCs); RNA interference; TCF/LEF reporter gene system; Wnt/ $\beta$ -catenin signaling.

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## Introduction

Human mesenchymal stem cells (hMSCs) increasingly attract the focus of both basic and clinical research approaches. They can be segregated from a variety of mesodermal tissues (Campagnoli et al., 2001; Miura et al., 2003; Schaffler and Buchler, 2007) and are capable of differentiating along the mesodermal lineage into bone, cartilage, fat, and muscle (Pittenger et al., 1999). More recently, it has been demonstrated by *in vivo* studies that hMSCs can transdifferentiate into endoderm-derived cells and cardiomyocytes (Toma et al., 2002; Sato et al., 2005). The central characteristics of stem cells are their capacity for self-renewal and differentiation (Weissman, 2000), which is tightly regulated within the stem cell niche (Spradling et al., 2001; Walker et al., 2009). The control of stem cell niches is emerging as a key role of MSCs in a broad range of tissues. In this context, MSC ablation was shown to disrupt hematopoiesis in mice (Raaijmakers et al., 2010). Moreover, there is accumulating evidence that hMSC are released from their perivascular location and, when activated, establish a regenerative microenvironment by secreting bioactive molecules (Crisan et al., 2008; Caplan and Correa, 2011). To fulfill these multiple functions in tissue repair processes, hMSCs are dependent on signals of the surrounding environment (Ho and Wagner, 2007; Uccelli et al., 2008).

In this context, several signal transduction pathways are involved in the maintenance of the stem cell fate or in triggering differentiation processes, in particular the Wnt/ $\beta$ -catenin signal transduction pathway (Reya et al.,

2003; Willert et al., 2003; Neth et al., 2006, 2007; Ling et al., 2009; Egea et al., 2012; Peröbner et al., 2012). In the absence of Wnt ligands,  $\beta$ -catenin is degraded by the destruction complex, which is formed by axin, adenomatosis polyposis coli (APC), glycogen-synthase-kinase-3 $\beta$ , and casein kinase 1 $\alpha$ . Binding of canonical Wnt ligands to a cell surface receptor complex consisting of a Frizzled receptor (Fzd) and the Wnt co-receptor low-density lipoprotein receptor-related protein 5 or 6 (LRP5 or LRP6) leads to the dissociation of the destruction complex, resulting in the stabilization of cytoplasmic  $\beta$ -catenin. After its translocation into the nucleus,  $\beta$ -catenin binds TCF/LEF transcription factors to activate Wnt/ $\beta$ -catenin target gene transcription (Nelson and Nusse, 2004; Kimelman and Xu, 2006), including cyclin D1 (Tetsu and McCormick, 1999) and Fzd7 (Willert et al., 2002).

It is of special interest that in the Wnt/ $\beta$ -catenin pathway, Fzds and LRP5 or LRP6 act together for the binding of Wnt ligands (Bhanot et al., 1996; He et al., 2004). In vertebrates, the Fzd family consists of 10 members (Fzd1–Fzd10) (Schulte and Bryja, 2007; Schulte, 2010). Fzds comprise seven transmembrane domains, three extracellular loops, and three intracellular loops being classified as a separate family in the group of G-protein-coupled receptors (Schulte and Bryja, 2007; Lagerstrom and Schioth, 2008; Schulte, 2010). The putative binding sites for the scaffold protein Dishevelled are located in the intracellular regions (Punchihewa et al., 2009), whereas the extracellularly located N-terminus is formed by a highly conserved cysteine-rich domain (CRD) serving as a binding site for Wnt ligands (Dann et al., 2001; Wang et al., 2006; Bourhis et al., 2010). The most obvious structural differences among the Fzd family members are found in the intracellular loops, and in particular in the C-terminus, suggesting that these regions play a major role in receptor-specific signaling (Wang et al., 2006).

As there is only fragmentary knowledge about the signaling function of Wnt receptors (particularly Fzd family members) at the cell surface of hMSCs, there is considerable interest for a detailed understanding of Wnt/ $\beta$ -catenin signaling at the receptor level of these cells to offer a new therapeutic route for a target-directed manipulation of Wnt/ $\beta$ -catenin signaling (Peröbner et al., 2012).

Concerning this background, we first performed expression profiling of these Wnt receptors and found that Fzd9 and Fzd10 were not expressed in hMSCs. Fzd3 exhibited very low expression levels, while the other Fzd family members showed high expression rates. Moreover, we found that the expression of Fzd1, Fzd6, and Fzd7 is positively correlated with the Wnt/ $\beta$ -catenin activation

status, whereas Fzd8 exhibited an inverse relation. RNA interference and ectopic expression studies in hMSCs carrying a TCF/LEF reporter gene system revealed that Fzd5, Fzd7, and Fzd8 play essential roles in mediating Wnt/ $\beta$ -catenin signaling. In this regard, our studies revealed that ectopic expression of Fzd5 can compensate the absence of Fzd7 and, conversely, that Fzd7 is able to restore Wnt/ $\beta$ -catenin signaling in hMSCs lacking Fzd5.

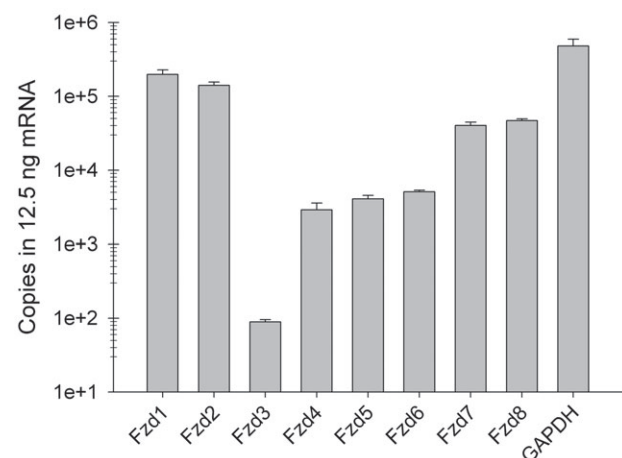
## Results

### Basal mRNA expression level of Fzds in hMSCs

To evaluate the basal mRNA expression of Fzds in hMSCs, comparative expression profiling was performed by qRT-PCR in hMSCs (Figure 1). The mRNA expression of the 10 Fzds can be subdivided into five major distinct groups. Accordingly, Fzd1 and Fzd2 exhibited the highest expression levels, followed by Fzd7 and Fzd8. Fzd4, Fzd5, and Fzd6 displayed lower expression rates. Fzd3 exhibited the lowest expression level (Figure 1). The mRNAs of Fzd9 and Fzd10 were not expressed in this setting.

### Regulation of Fzd expression by Wnt/ $\beta$ -catenin signaling

In the next step, we analyzed whether the expression of the Fzds is regulated through the Wnt/ $\beta$ -catenin pathway



**Figure 1** Basal Fzd expression pattern on mRNA level in hMSCs. Quantitative RT-PCR was carried out using the UPL system in combination with a LightCycler. For the absolute determination of the copy numbers of each Fzd, a standard curve with the respective Fzd plasmid vector dilution was generated.

itself. For activation of the pathway, hMSCs were either stimulated with Wnt3a or transfected with siRNAs against APC, which resembles an essential component of the  $\beta$ -catenin destruction complex. Conversely, for the inhibition of Wnt/ $\beta$ -catenin signaling, RNAi against  $\beta$ -catenin was performed. In all three approaches, qRT-PCR of the Fzd expression profile was performed at days 1, 3, and 7.

The results provide substantial evidence that Fzd1, Fzd6, and Fzd8 are regulated by Wnt/ $\beta$ -catenin signaling (Figure 2 A–L). Concerning Fzd1, Fzd6, and Fzd7, our data suggest a positive correlation between the respective Fzd expression level and the Wnt/ $\beta$ -catenin activation status in hMSCs (upregulation after Wnt3a stimulation or APC knockdown and repression upon  $\beta$ -catenin knockdown)

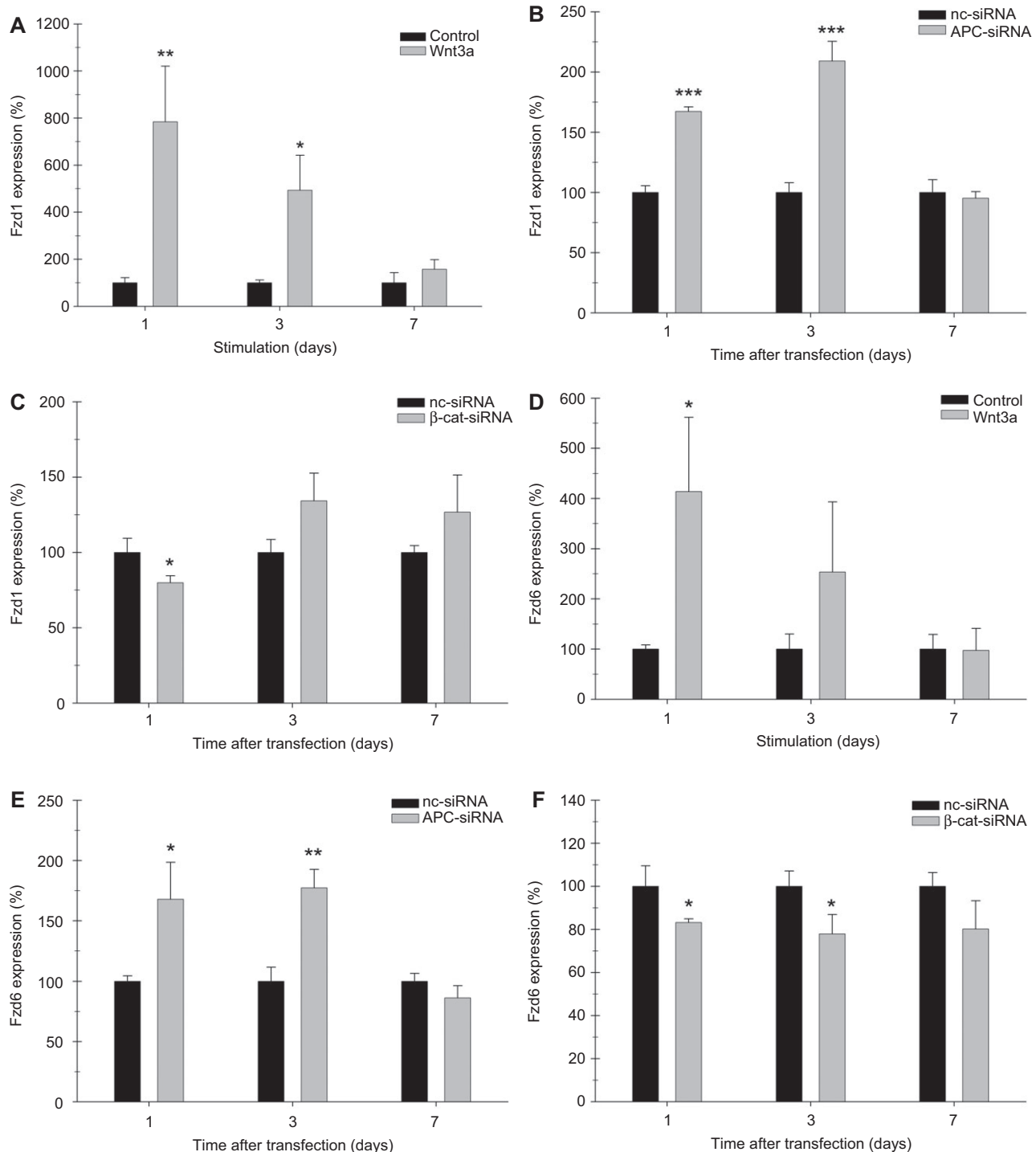
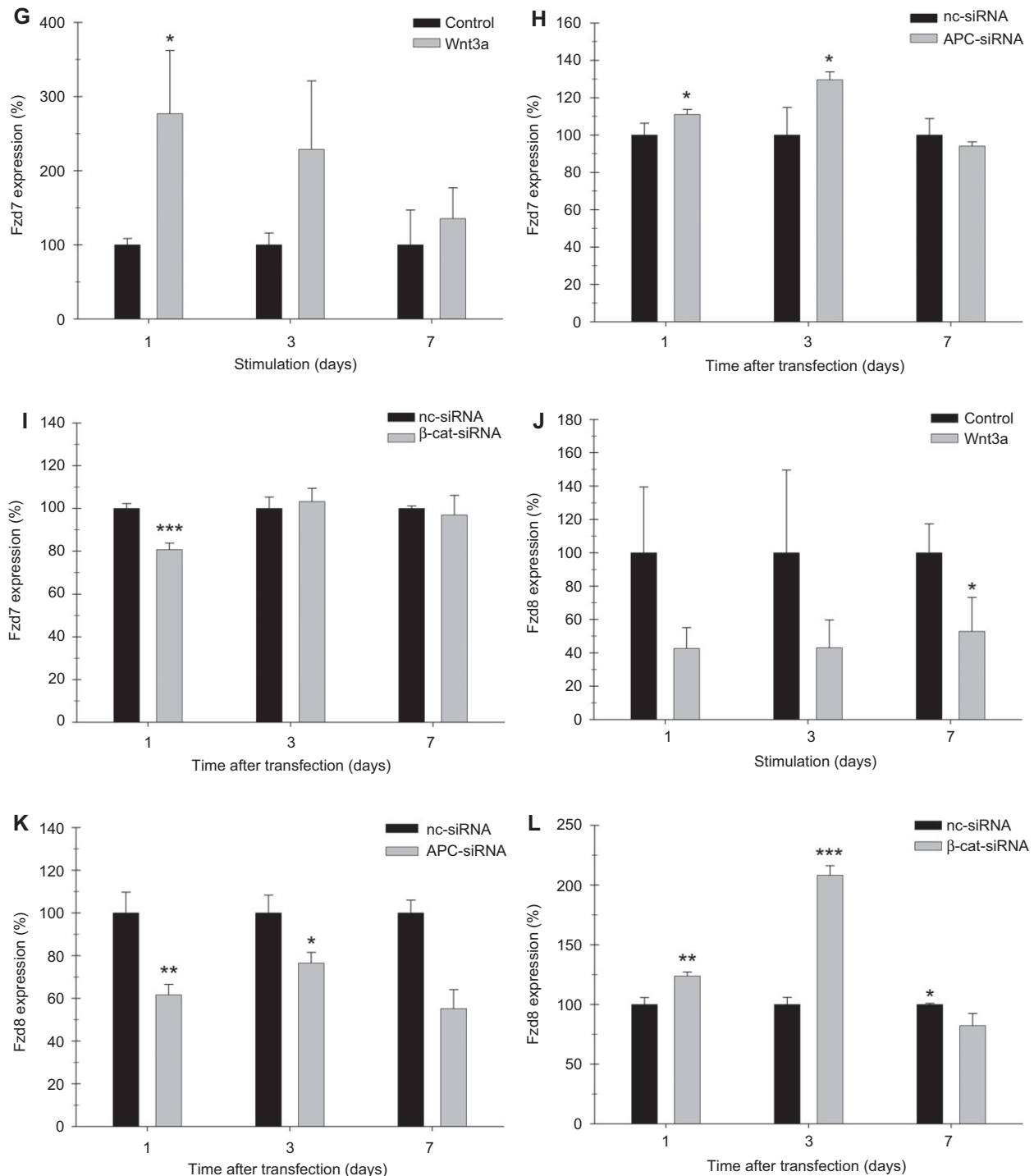


Figure 2

(Figure 2 continued)

**Figure 2** Regulation of Fzd expression by Wnt/ $\beta$ -catenin signaling.

For activation of the pathway, hMSCs were stimulated with Wnt3a (A, D, G, J) or transfected with APC-siRNA (B, E, H, K). Inhibition of the pathway was achieved by RNAi against  $\beta$ -catenin (C, F, I, L). qRT-PCR of the respective Fzds was performed 1, 3, and 7 days after Wnt3a stimulation or transfection with the respective siRNA. (A–C) Fzd1-mRNA expression; (D–F) Fzd6-mRNA expression; (G–I) Fzd7-mRNA expression; (J–L) Fzd8-mRNA expression.

(Figure 2A–I), while the mRNA expression of Fzd8 indicates a negative interrelation (repression after Wnt3a stimulation or APC knockdown and upregulation upon

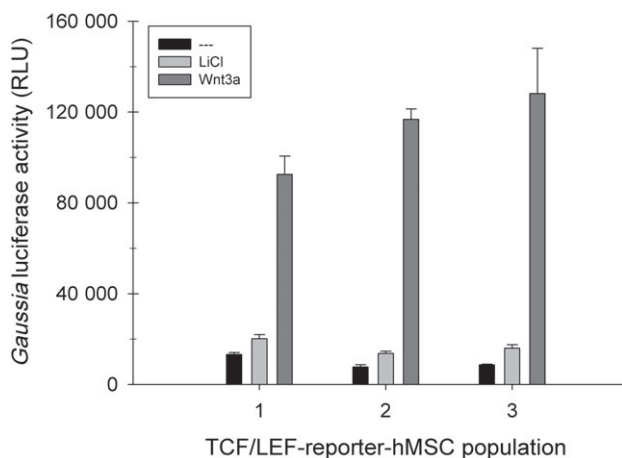
$\beta$ -catenin knockdown) (Figure 2J–L). The mRNA expression levels of the other Fzds were also studied; however, we observed no regulation by the Wnt/ $\beta$ -catenin pathway.

## Signaling capacity of TCF/LEF-reporter-hMSCs

To determine the signaling capacity of stably pN3-Bar-GLuc-transfected hMSCs (TCF/LEF-reporter-hMSCs), stimulation with LiCl and Wnt3a was carried out. The *Gaussia* luciferase activities of three different, representative TCF/LEF-reporter-hMSC populations are depicted in Figure 3. Stimulation with 4 mM LiCl resulted in a moderate activation of Wnt/ $\beta$ -catenin signaling (1.5–1.9-fold), whereas application of 75 ng/ml Wnt3a led to a strong activation of  $\beta$ -catenin-mediated transcription (7.0–15.1-fold). As the respective control, hMSCs were stably transfected with pN3-fuBar-GLuc (negative control). These pN3-fuBar-GLuc-hMSCs exhibited no induction of *Gaussia* luciferase activity upon activation of the Wnt/ $\beta$ -catenin pathway (data not shown).

## RNAi against Fzds in TCF/LEF-reporter-hMSCs

To investigate the impact of Fzd-mediated signaling on the Wnt/ $\beta$ -catenin pathway in a quantitative manner, RNAi against the expressed Fzds was performed in TCF/LEF-reporter-hMSCs. Furthermore, APC- and  $\beta$ -catenin-siRNA transfections served as controls for the activation and inhibition of Wnt/ $\beta$ -catenin signaling. The knockdown efficiencies for Fzd1, Fzd2, Fzd3, Fzd4, Fzd5, Fzd6, Fzd7, Fzd8, APC, and  $\beta$ -catenin were determined on mRNA level at

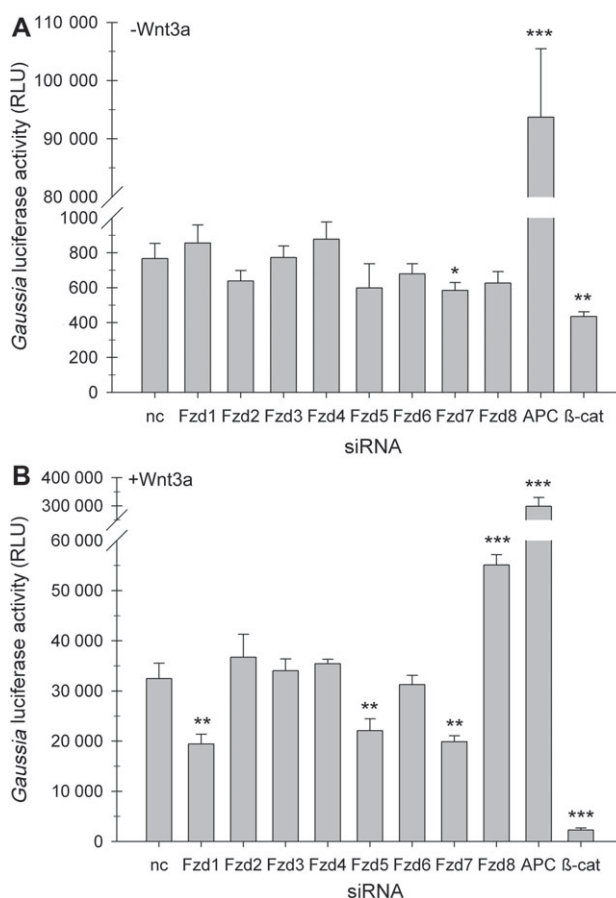


**Figure 3** Absolute *Gaussia* luciferase activity values of different TCF/LEF-reporter-hMSC populations. After the stable transfection of hMSCs with pN3-Bar-GLuc containing 12 TCF/LEF consensus sequences in front of the reporter gene *Gaussia* luciferase, the obtained hMSC populations were analyzed with regard to their Wnt/ $\beta$ -catenin signaling capacity by measurement of the *Gaussia* luciferase activity (relative luminescence units, RLUs). The different populations were treated with either 4 mM LiCl or 75 ng/ml Wnt3a. Three representative populations, showing the signaling activity at day 5, are depicted.

days 1 and 7 after siRNA transfection, and are summarized in Table S1 and indicated as percentages of knockdown.

Under non-stimulatory conditions, only RNAi against Fzd7 revealed a significant impairment (24%) of Wnt/ $\beta$ -catenin signaling in hMSCs (Figure 4A). RNAi against Fzd2, Fzd5, Fzd6, and Fzd8 showed a slight but not significant decrease in *Gaussia* luciferase activity under these conditions. The respective controls came along with the expected changes in Wnt/ $\beta$ -catenin signaling. Namely, RNAi against APC resulted in a massive induction of the reporter protein activity, while the knockdown of  $\beta$ -catenin resulted in a remarkable reduction of Wnt/ $\beta$ -catenin signaling in hMSCs (Figure 4A).

This situation considerably changed under Wnt3a-stimulatory conditions (Figure 4B). RNAi against Fzd1,



**Figure 4** RNAi against Fzds in TCF/LEF-reporter-hMSCs. TCF/LEF-reporter-hMSCs were transfected with the respective siRNAs against each of the Fzds,  $\beta$ -catenin, and APC. The activity of the reporter protein *Gaussia* luciferase (RLU) was analyzed 7 days after transfection under non-stimulatory (A) as well as Wnt3a-stimulatory (B) conditions. Fzd-, APC-, and  $\beta$ -catenin-transfected TCF/LEF-reporter-hMSCs were compared with nc-siRNA-transfected TCF/LEF-reporter-hMSCs.



Fzd5, and Fzd7 caused a marked decrease in Wnt/ $\beta$ -catenin signaling (reduction of 40%, 32%, and 39%), whereas abrogation of Fzd8 resulted in a significant increase of the reporter gene activity. The knockdown of Fzd2, Fzd3, Fzd4, and Fzd6 had no influence on  $\beta$ -catenin-mediated signaling in hMSCs in the presence of Wnt3a (Figure 4B). These results point to the fact that only Fzd5 and Fzd7 are involved in the activation of  $\beta$ -catenin-mediated signaling under both basal and Wnt3a-stimulatory conditions.

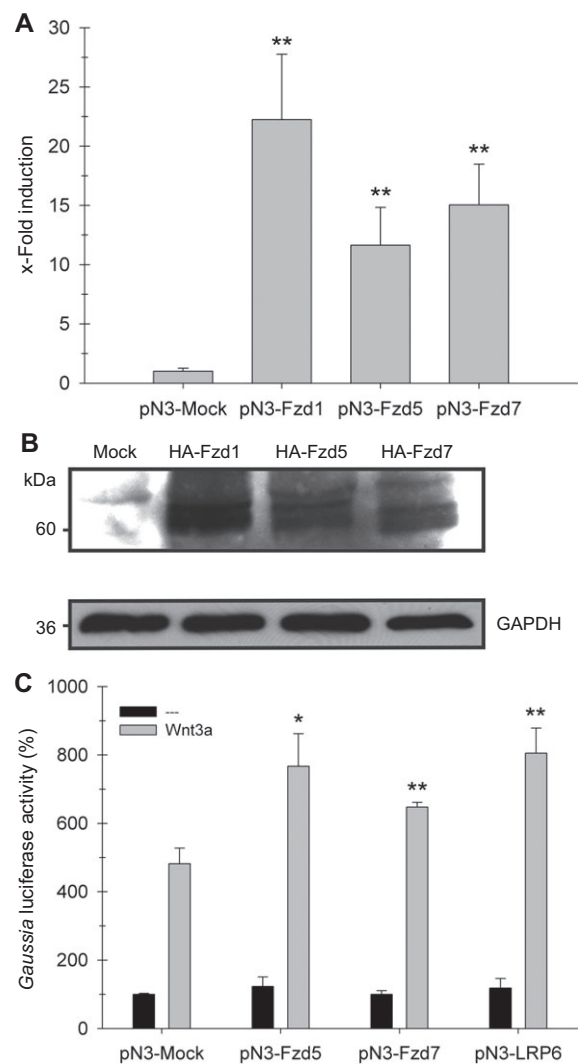
### Ectopic expression of Fzd1, Fzd5, and Fzd7 in hMSCs

On the basis of the fact that RNAi against Fzd1, Fzd5, and Fzd7 resulted in an impaired  $\beta$ -catenin-mediated signaling under Wnt3a-stimulatory conditions, we analyzed whether the ectopic expression of these receptors would lead to adverse effects. For this purpose, hMSCs were transfected with the Fzd expression plasmids pN3-Fzd1, pN3-Fzd5, or pN3-Fzd7. The Mock vector backbone (pN3-Mock) served as the respective control. Ectopic expression was examined on mRNA level by qRT-PCR (Figure 5A) and on protein level by Western blot analysis (Figure 5B). At day 1, we observed a significant mRNA upregulation of Fzd1, Fzd5, and Fzd7 (12-, 22-, and 15-fold, respectively) (Figure 5A). The ectopic expression of the respective Fzd proteins was determined 2 days after transfection of hMSCs with pN3-Fzd1, pN3-Fzd5, and pN3-Fzd7 by application of HA-tag-directed antibodies (Figure 5B).

To study the influence of the ectopic expression of these receptors on Wnt/ $\beta$ -catenin signaling, transient co-transfections of the Fzd expression plasmids together with the TCF/LEF reporter gene plasmid pN3-Bar-GLuc were carried out under basal and Wnt3a-stimulatory conditions. Ectopic expression under Wnt3a-stimulatory conditions revealed that Fzd5 and Fzd7 enhance Wnt/ $\beta$ -catenin-dependent signal transduction in hMSCs when compared with the Mock control (Figure 5C). These increases in *Gaussia* luciferase activity were similar to that after ectopic expression of LRP6 (Peröbner et al., 2012). In contrast, overexpression of Fzd1 did not result in any changes of the reporter protein activity (data not shown).

### Rescue of Fzd5- and Fzd7-mediated signaling in hMSCs

On the basis of the results that the knockdown of Fzd5 and Fzd7 led to a decrease in Wnt/ $\beta$ -catenin signaling under



**Figure 5** Ectopic expression of Fzd1, Fzd5, and Fzd7 in hMSCs. hMSCs were transfected with pN3-Fzd1, pN3-Fzd5, pN3-Fzd7, or pN3-LRP6, and ectopic mRNA expression was determined in comparison with pN3-Mock (control)-transfected hMSCs (A). For the analysis of the ectopic protein expression of the Fzds, hMSCs were transfected with the tagged variants of Fzd1, Fzd5, and Fzd7. Detection was performed with an anti-HA-antibody 2 days after transfection of the hMSCs (B). As a loading control, GAPDH was detected using an anti-GAPDH antibody (B). For studying the influence of the ectopic expression of these Fzds on  $\beta$ -catenin-mediated signaling, hMSCs were transfected with pN3-Mock (control), pN3-Fzd5, pN3-Fzd7, or pN3-LRP6 together with the TCF/LEF reporter gene plasmid pN3-Bar-GLuc. Quantification of the *Gaussia* luciferase activity was carried out under basal as well as Wnt3a-stimulatory conditions. Statistical significance was calculated in the non-stimulated samples in relation to pN3-Mock-transfected hMSCs (-Wnt3a) and in the Wnt3a-stimulated samples in relation to pN3-Mock-transfected hMSCs (+Wnt3a). Moreover, pN3-Mock-transfected hMSCs (-Wnt3a) were set as 100% (C).

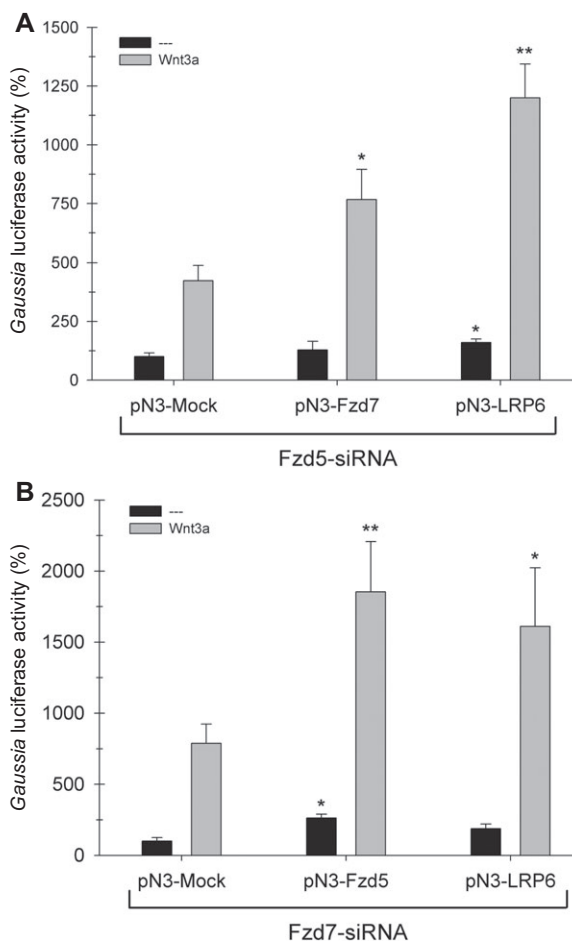
basal and even more under Wnt3a-stimulatory conditions, we investigated whether the signaling function of Fzd5 or Fzd7 can be restored by ectopic expression of the

respective other Fzd. Moreover, we analyzed whether the loss of Fzd5 or Fzd7 can be compensated by the ectopic expression of the Wnt co-receptor LRP6.

For this purpose, the expression of Fzd5 or Fzd7 was repressed by transfection of the respective siRNAs. Five days later, these hMSCs were co-transfected with pN3-Bar-GLuc and pN3-Fzd5 (in Fzd7-knockdown hMSCs), pN3-Fzd7 (in Fzd5-knockdown hMSCs), pN3-LRP6, or the respective Mock-vector control (in Fzd5- and Fzd7-knockdown hMSCs). The effects on Wnt/ $\beta$ -catenin signaling were analyzed under basal and Wnt3a-stimulatory conditions. The results revealed that in hMSCs carrying a knockdown of Fzd5, the ectopic expression of Fzd7 can compensate for the lack of Fzd5 (Figure 6A). Conversely, the ectopic expression of Fzd5 in Fzd7-knockdown hMSCs also resulted in a rescue of the Wnt/ $\beta$ -catenin signaling capacity (Figure 6B). It is noteworthy that the ectopic expression of the Wnt co-receptor LRP6 is also able to restore Wnt/ $\beta$ -catenin signaling in hMSCs carrying a knockdown of either Fzd5 or Fzd7 (Figure 6A, B). In contrast, the ectopic expression of Fzd1 in hMSCs carrying a knockdown of Fzd5 or Fzd7 did not result in a rescue of Wnt/ $\beta$ -catenin signaling in hMSCs (data not shown).

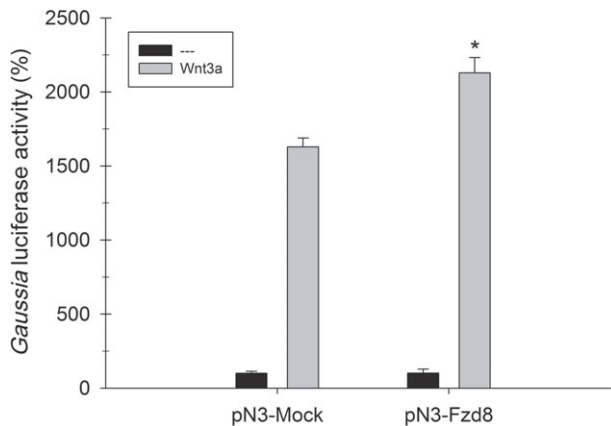
### Ectopic expression of Fzd8 in hMSCs

As the knockdown of Fzd8 resulted in an enhanced *Gaussia* luciferase in TCF/LEF-reporter-hMSCs under Wnt3a stimulatory conditions (Figure 4B), we analyzed whether overexpression of Fzd8 might result in the opposite effect. For this purpose, hMSCs were co-transfected with pN3-Fzd8 and pN3-Bar-GLuc, while the co-transfection of pN3-Mock and pN3-Bar-GLuc served as control. The results revealed that under Wnt3a-stimulatory conditions, overexpression of Fzd8 resulted in an enhanced *Gaussia* luciferase activity. In contrast, overexpression of Fzd8 under basal conditions did not result in any changes in the *Gaussia* luciferase activity (Figure 7). Owing to these unexpected results (induction of the *Gaussia* luciferase after knockdown and overexpression of Fzd8 under Wnt3a-stimulatory conditions), we performed a detailed investigation of the mRNA transcripts of Fzd8 in hMSCs by using RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE). Thereby, we detected beyond the transcription start site at position 1 additional start sites at the positions +15, +16, and +17. As the first start codon (ATG) begins at +5, the +15, +16, and +17 mRNA transcripts would result in an N-terminal-truncated



**Figure 6** Rescue of Fzd5- and Fzd7-mediated signaling in hMSCs. The expression of Fzd5 and Fzd7 was repressed by the transfection of the respective siRNAs (A, B). Fzd5-knockdown hMSCs were co-transfected with pN3-Bar-GLuc and pN3-Fzd7 or pN3-LRP6 (A), while hMSCs carrying a knockdown of Fzd7 were co-transfected with pN3-Bar-GLuc and pN3-Fzd5 or pN3-LRP6 (B). Co-transfection of pN3-Bar-GLuc with pN3-Mock served as the respective control in all three approaches. *Gaussia* luciferase activity was determined under basal as well as Wnt3a-stimulatory conditions. Statistical significance was calculated in the non-stimulated samples in relation to pN3-Mock-transfected hMSCs (-Wnt3a) and in the Wnt3a-stimulated samples in relation to pN3-Mock-transfected hMSCs (+Wnt3a). Moreover, pN3-Mock-transfected hMSCs (-Wnt3a) were set to 100% as the respective reference.

Fzd8 protein. Because the next in-frame start codon is located 156 bp downstream of the first ATG, this would result in a 52-amino-acid N-terminal truncation of Fzd8. On the basis of these observations, we cloned this truncated version of Fzd8. The first transfection experiments revealed that, in contrast to full-length Fzd8, this truncated Fzd8 isoform exhibited no induction of the *Gaussia* luciferase under basal and Wnt3a-stimulatory conditions (data not shown).



**Figure 7** Ectopic expression of Fzd8 in hMSCs.

For studying the influence of the ectopic expression of Fzd8 on  $\beta$ -catenin-mediated signaling, hMSCs were transfected with pN3-Mock (control) or pN3-Fzd8 together with the TCF/LEF reporter gene plasmid pN3-Bar-GLuc. Quantification of the *Gaussia* luciferase activity was carried out under basal as well as Wnt3a-stimulatory conditions. Statistical significance was calculated in the non-stimulated samples in relation to pN3-Mock-transfected hMSCs (-Wnt3a) and in the Wnt3a-stimulated samples in relation to pN3-Mock-transfected hMSCs (+Wnt3a). Moreover, pN3-Mock-transfected hMSCs (-Wnt3a) were set as 100%.

## Discussion

As the Wnt/ $\beta$ -catenin signal transduction pathway is critically involved in both the regulation of stem cell attributes and in driving malignant processes, modulation of Wnt signaling (e.g., regulation of Fzd activity) may offer a novel route in enhancing tissue repair as well as in the prevention of numerous cancer entities (Ettenberg et al., 2010; Gong et al., 2010). Thus, understanding of the initial steps of Wnt signaling in hMSCs at the receptor level is of fundamental importance. For an overview, the essential findings of our work are summarized in Table 1.

### Different expression levels of Fzds

In our qRT-PCR approach, remarkable differences in the receptor expression levels were observed. While Fzd1, Fzd2, Fzd4, Fzd5, Fzd6, Fzd7, and Fzd8 were expressed in quite high amounts, Fzd3 displayed much lower expression levels, while Fzd9 and Fzd10 were not expressed. In 2004 a qualitative approach on mRNA level with respect to Fzd family members has already been accomplished in hMSCs, although only a subset of Fzds could be detected in this work. Among those, Fzd2, Fzd3, Fzd4, Fzd5, and Fzd6 were expressed, whereas Fzd1, Fzd7, Fzd8, and

|       | Basal Fzd expression | Regulation by Wnt3a | Responsiveness to Wnt3a | TCF/LEF binding sites |
|-------|----------------------|---------------------|-------------------------|-----------------------|
| Fzd1  | ++++                 | +++                 | +++                     | 10                    |
| Fzd2  | ++++                 | n.r.                | n.r.                    | n.a.                  |
| Fzd3  | +                    | n.r.                | n.r.                    | n.a.                  |
| Fzd4  | ++                   | n.r.                | n.r.                    | n.a.                  |
| Fzd5  | ++                   | n.r.                | +++                     | n.a.                  |
| Fzd6  | ++                   | +++                 | n.r.                    | 9                     |
| Fzd7  | +++                  | +++                 | +++                     | 16                    |
| Fzd8  | +++                  | ---                 | ++                      | 2                     |
| Fzd9  | n.e.                 | n.a.                | n.a.                    | n.a.                  |
| Fzd10 | n.e.                 | n.a.                | n.a.                    | n.a.                  |

**Table 1** Specific characteristics of Fzds in hMSCs.

n.a., not analyzed; n.e., not expressed; n.r., no regulation/responsiveness.

Fzd10 were not detected in this setting (Etheridge et al., 2004). Another Fzd evaluation in hMSCs revealed the mRNA expression of nine Fzds (Okoye et al., 2008). However, Fzd8 was not detected in this study, which is in contrast to our findings, where Fzd8 was expressed at high levels in hMSCs. It is of special interest that our finding on Fzd8 expression in hMSCs is corroborated by a semiquantitative RT-PCR study where Fzd8 expression was found at high levels in hMSCs (Boland et al., 2004). Moreover, this study showed, in line with our results, low expression of Fzd3. Fzd9 was also not detected in this semiquantitative RT-PCR approach (Boland et al., 2004).

In a recently published study, several hMSC lines had been derived from human placenta and examined concerning the expression of their cell surface markers and their ability to differentiate toward mesenchymal lineages (Tran et al., 2011). The authors found that the expression of Fzd9/CD349 was positive in a fraction of placenta-derived hMSCs (PL-hMSCs). Transplantation experiments in mice in ischemic regions revealed that only Fzd9-negative PL-hMSCs have a greater ability to induce re-endothelialization and new vessel formation when compared with Fzd9-positive PL-hMSCs. In contrast, both the Fzd9-positive and -negative fractions facilitated new bone calcification in fractured femurs. On the basis of these findings, the authors propose that Fzd9 might be used as a specialized MSC marker for arteriogenesis and angiogenesis (Tran et al., 2011).

In adult human periodontal ligament (PDL), a population of stem cells (PDL-MSCs) was identified with the ability to differentiate into osteoblasts and adipocytes. A subset of these cells also showed Fzd9/CD349 receptor expression on its surface (Trubiani et al., 2010). While at the second passage Fzd9 was clearly expressed, it was downregulated in subsequent passages. Comparative



growth analysis of Fzd9<sup>+</sup> and Fzd9<sup>-</sup> PDL-MSCs suggested a difference in proliferation between these two subpopulations. In this study, Fzd9<sup>-</sup> cells exhibited a higher proliferation capacity when compared with the Fzd9<sup>+</sup> population (Trubiani et al., 2010).

These findings in PL-hMSCs and PDC-hMSCs are at least partially in contrast to our findings in bone marrow-derived hMSCs where we did not detect any Fzd9 expression. This discrepancy might be due to the different tissue origins of these hMSC isolates.

## Regulation of Fzd expression by Wnt3a/ $\beta$ -catenin signaling

Furthermore, our experiments revealed that in hMSCs, the mRNA expression of Fzd1, Fzd6, Fzd7, and Fzd8 is regulated by Wnt/ $\beta$ -catenin signaling. While the mRNA expression of Fzd1, Fzd6, and Fzd7 exhibited a positive correlation with the Wnt/ $\beta$ -catenin status in hMSCs, Fzd8 mRNA expression displayed an inverse relation. Up to now, only Fzd7 has been reported to be a positive Wnt target gene, which has been demonstrated in human embryonic carcinoma cells (Willert et al., 2002). By comparing the global gene expression profiles of undifferentiated, differentiated, and dedifferentiated hMSCs in three mesodermal lineages, a number of ‘stemness’ and ‘differentiation’ genes was identified that might be essential to maintain adult stem cell multipotency and to drive lineage-specific commitment (Song et al., 2006). Using siRNA gene inactivation, the authors demonstrate that among others, particularly Fzd7 promoted cell survival, as well as the commitment of hMSCs for differentiation into the chondrogenic lineage, while suppressing osteogenesis. However, the influence of Fzd7 on Wnt/ $\beta$ -catenin signaling was not analyzed in this study.

Moreover, it has been reported that a cross-talk between Wnt signaling pathways in hMSCs leads to functional antagonism during osteogenic differentiation (Baksh et al., 2007). In this context, it has been demonstrated that the overexpression of LRP5 leads to changes in the Fzd expression profile. Among the investigated Fzds (Fzd1, Fzd3, Fzd4, Fzd6, and Fzd7), it was found that overexpression of LRP5, which came along with the activation of the Wnt/ $\beta$ -catenin pathway, resulted in an upregulation of Fzd1 and Fzd7 under basal, and even more pronounced, under osteogenic culture conditions (Baksh et al., 2007). These results are in line with our findings that Fzd1 and Fzd7 are upregulated upon stimulation with Wnt3a or after RNAi against APC. Beyond that, we also found under basal conditions a positive correlation between the activation

of the Wnt/ $\beta$ -catenin pathway and Fzd6 expression, and a negative relation between  $\beta$ -catenin-mediated signaling and Fzd8 expression, which had not been reported by Baksh et al.

To obtain a deeper insight into these regulation mechanisms, *in silico* promoter studies 3000 bp upstream of the supposed transcription start site of each Wnt3a/ $\beta$ -catenin-regulated Fzd were accomplished and revealed 10 TCF/LEF binding sites (consensus sequence: WWCAAAG; van de Wetering et al., 1997; Tetsu and McCormick, 1999) in the case of Fzd1, 9 TCF/LEF consensus sequences concerning Fzd6, and 16 putative TCF/LEF binding sites with regard to Fzd7. In contrast, the *in silico* promoter analysis of Fzd8 revealed only two TCF/LEF consensus sequences, both, however, 1600 and 2900 bp upstream of the transcription start site. The presence of numerous consensus sequences for a putative experimental data of TCF/LEF transcription factors substantiates our findings that besides Fzd7, also Fzd1 and Fzd6 are positively regulated by Wnt/ $\beta$ -catenin signaling in hMSCs. Besides our experimental findings of changes in Fzd expression upon Wnt3a stimulation, APC knockdown or  $\beta$ -catenin knockdown, and the fact that we found numerous TCF/LEF consensus sequences by *in silico* analysis in the promoter regions of Fzd1, Fzd6, and Fzd7, we cannot exclude that in this experimental setting these Fzds might not be exclusively regulated as direct Wnt targets.

It is noteworthy that Fzd8 may represent a new candidate for a negatively regulated Wnt/ $\beta$ -catenin target gene. Up to now, only a few genes have been identified in humans that are repressed by Wnt/ $\beta$ -catenin signaling comprising, e.g., Sox9 in mesenchymal cells (Hill et al., 2005), p16<sup>INK4a</sup> in melanocytes, and Nanog in embryonic stem cells (Pereira et al., 2006). The exact mechanism regulating the repression of Wnt target genes on the one hand and a coincidental activation of other Wnt target genes on the other hand is only partially understood. Different members of the TCF/LEF family can have activating as well as inhibitory functions concerning selected Wnt target genes (Standley et al., 2006; Hoppler and Kavanagh, 2007). In this context, the DNA consensus sequences, where TCF/LEF transcription factors bind, play an important role with regard to the activation or repression of Wnt target genes as outlined recently (Blauwkamp et al., 2008). Noteworthy, DNA consensus sequences for repressing Wnt/ $\beta$ -catenin-mediated gene expression were recently deciphered in *Drosophila* (Blauwkamp et al., 2008). However, orthologous sequences in vertebrates have not been identified up to now (Hoverter and Waterman, 2008).

Our findings that several members of the Fzd family are subjected to the regulation of the Wnt/ $\beta$ -catenin signaling pathway through positive or negative feedback loops is of fundamental importance, as Fzds might represent future drug targets in therapeutic approaches not only in neoplastic diseases (Barker and Clevers, 2006) but also in supporting regenerative processes (Moon et al., 2004). With respect to the latter, many components of the Wnt/ $\beta$ -catenin pathway, including Fzds, are involved in the complex interplay to strike a balance between activation and inhibition of this signal transduction cascade in regeneration and development.

### Responsiveness of Fzds to Wnt3a/ $\beta$ -catenin signaling

One major barrier for studying Wnt/ $\beta$ -catenin signaling in hMSCs over a prolonged time period in a highly sensitive and, in particular, specific manner, is the lack of an effective  $\beta$ -catenin-dependent reporter gene system in this stem cell population. Thus, we stably transfected hMSCs with a TCF/LEF reporter gene plasmid encoding the *Gaussia* luciferase to generate TCF/LEF-reporter-hMSCs. Numerous TCF/LEF-reporter-hMSC populations were generated, and the functionality of this reporter system was demonstrated by the activation of Wnt/ $\beta$ -catenin signaling either by the application of Wnt3a or LiCl. On the basis of the fact that *Gaussia* luciferase is secreted into the cell culture supernatant, this system allows monitoring of minimal changes in  $\beta$ -catenin-mediated expression of the reporter protein *Gaussia* luciferase in the respective TCF/LEF-reporter-hMSCs.

For unraveling the function of Fzds concerning Wnt/ $\beta$ -catenin signaling, we performed RNAi against the expressed Fzds in these TCF/LEF-reporter-hMSCs. We found that under basal and Wnt3a-stimulatory conditions, Fzd5 and Fzd7 fulfill signal-activating functions. These findings could be corroborated by our ectopic expression studies of Fzd5 and Fzd7, which resulted in an enhanced *Gaussia* luciferase activity. In this experiment, pN3-Mock-transfected hMSCs also exhibited a *Gaussia* luciferase induction upon Wnt3a stimulation. This might be due to the fact that pN3-Mock-transfected hMSCs, similar to non-transfected hMSCs, express endogenous levels of Fzds, LRP5, and LRP6 (Etheridge et al., 2004; Okoye et al., 2008; Peröbner et al., 2012). Thus, with the stimulation of Wnt3a, an induction of the *Gaussia* luciferase activity can also be expected in pN3-Mock-transfected hMSCs.

Moreover, the rescue experiments also revealed that the absence of Fzd5 could be compensated by an enhanced

expression of Fzd7. Conversely, the knockdown of Fzd7 could be counterbalanced by the ectopic expression of Fzd5. In hMSCs carrying a knockdown of Fzd5 (Figure 6A) or Fzd7 (Figure 6B), stimulation with Wnt3a results in a marked induction of the *Gaussia* luciferase activity also in pN3-Mock-transfected hMSCs. This might be explainable by the fact that the remaining Wnt3a receptors (Fzd7 in the Fzd5-knockdown or Fzd5 in the Fzd7-knockdown) together with LRP5 and/or LRP6 are still able to transduce the Wnt3a signal from the membrane into the cell. Moreover, increasing the amounts of Fzd7 (Figure 6A), Fzd5 (Figure 6B), or LRP6 (Figure 6A,B) by transfection with pN3-Fzd7, pN3-Fzd5, or pN3-LRP6 resulted, under Wnt3a-stimulatory conditions, in a marked increase of the *Gaussia* luciferase activity when compared with pN3-Mock-transfected hMSCs. Under basal conditions without Wnt3a, a similar tendency was observed but was not statistically significant.

Concerning the function of Fzd8, we found that RNAi against Fzd8 and also Fzd8 overexpression resulted in an enhanced Wnt/ $\beta$ -catenin signaling in hMSCs. To obtain a deeper insight into these unexpected results, we analyzed whether there might exist additional isoforms of Fzd8. Transcriptional start site mapping revealed substantial evidence for an additional isoform of Fzd8. This Fzd8 variant lacks the first 52 amino acids, which would result in an N-terminal-truncated Fzd8. Interestingly, this isoform exhibited no Wnt3a/ $\beta$ -catenin signaling capacity. In this context, it has been reported that the N-terminal region of the Fzd8-CRD is of fundamental importance for the binding of Wnt ligands (Dann et al., 2001). Thus, it becomes feasible that in hMSCs, two different isoforms are expressed (full-length and truncated Fzd8). However, only the full-length isoform is able to bind Wnt3a for initiating Wnt/ $\beta$ -catenin signaling, while the truncated isoform cannot induce the Wnt3a/ $\beta$ -catenin cascade. Further experiments have to be done to obtain a more detailed picture of the molecular function of the truncated Fzd8 and if this isoform may act as a dominant-negative Fzd8 receptor.

Our findings are supported by a recently published study in which the signaling potentials of Fzd5, Fzd7, and Fzd8 were investigated through the determination of the binding activities of their CRDs to Wnt3a (Kemp et al., 2007). The authors demonstrated that all three Fzd CRDs can block Wnt3a activity in L cells, indicating that the CRDs of Fzd5, Fzd7, and Fzd8 are able to bind Wnt3a. In this context, it has been demonstrated by an ELISA-based technique that besides Wnt7a, Wnt3a can also bind to the CRD of Fzd5, resulting in  $\beta$ -catenin signaling in endometrial cells (Carmon and Loose, 2010). With regard to Fzd7, it has been shown that this receptor contributes to self-renewal

in human embryonic stem cells (Melchior et al., 2008); however, the signaling mechanism and the responsible Wnt ligand(s) have not been identified thus far.

Thus, when combining the data, Fzd1 and Fzd7 are positively regulated by the Wnt/ $\beta$ -catenin pathway and function additionally as Wnt3a receptors in hMSCs. Consequently, an elevated Wnt/ $\beta$ -catenin level would lead to an increased expression of Fzd1 and Fzd7, which might also result, at least in part, in an enhanced sensitivity to Wnt3a. Fzd5 acts as Wnt3a receptor in hMSCs, but shows no regulation by Wnt/ $\beta$ -catenin signaling. Conversely, Fzd6 is positively regulated by the Wnt/ $\beta$ -catenin pathway but does not act as Wnt3a receptor in hMSCs. In contrast, Fzd8 is negatively regulated by Wnt/ $\beta$ -catenin signaling and its full-length isoform functions as a Wnt3a receptor in hMSCs (see Table 1).

## Conclusion

We have demonstrated in our study that numerous Fzds are expressed in hMSCs, and that Fzd1, Fzd6, Fzd7, and Fzd8 are regulated by the Wnt/ $\beta$ -catenin pathway. By using a highly sensitive TCF/LEF reporter gene system, we could demonstrate that Fzd1, Fzd5, Fzd7, and Fzd8 play essential roles in Wnt/ $\beta$ -catenin signaling in hMSCs. Moreover, there exists a functional redundancy between Fzd5 and Fzd7.

## Materials and methods

### Cell culture and differentiation of hMSCs

Cryopreserved hMSCs originated from a 27-year-old man (donor 5064L) and were kindly provided by the laboratory of Dr. D. Prockop, Tulane University, New Orleans, LA, USA. After the drawing of bone marrow aspirate, mononuclear cells were separated by density centrifugation. The cells were plated to obtain adherent hMSCs, which were harvested when the cells reached a confluence of 70–80% (passage 0). The characterization of these hMSC isolates was carried out by Prockop's laboratory according to the protocol of Sekiya et al. (2002). Cells were negative with regard to the expression of CD34, CD36, CD45, and CD117, and positive for CD44, CD90, CD166, CD105, CD29, CD49c, CD147, and CD59 when grown in  $\alpha$ -minimum essential medium ( $\alpha$ MEM) (PAA, Pasching, Austria) containing 2 mM L-glutamine, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and 16.5% FBS (Sigma, St. Louis, MO, USA). In our laboratory, hMSCs were cultivated at 37°C in a humidified atmosphere containing 95% air and 5% CO<sub>2</sub>. The medium was exchanged twice a week, and cells were used for further subculturing or cryopreservation at 90% confluence. For stimulation studies, recombinant Wnt3a (75 ng/ml) (R&D Systems,

Minneapolis, MN, USA) was applied. The hMSC differentiation capacity along the mesodermal lineages was demonstrated as previously described (Hoelters et al., 2005).

### RNA isolation and quantitative RT-PCR of Fzds, APC, and $\beta$ -catenin

Total RNA was isolated from cultured hMSCs using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The amount of RNA was determined photometrically (OD<sub>260 nm</sub>) using a Nanodrop photometer (PiqLab, Erlangen, Germany). cDNA synthesis was performed from 0.25  $\mu$ g total RNA, using the Quantitect Reverse Transcription Kit (Qiagen) as recommended by the supplier. Quantitative expression profiling of the Fzds was carried out with a LightCycler (Roche, Mannheim, Germany) using the Universal Probe Library System (UPL) in combination with the LightCycler Taq Man Master Kit (Roche). In the PCR procedure, the following substances were applied: 1  $\mu$ l cDNA as a template, 0.2  $\mu$ l of each primer, 0.1  $\mu$ l of the corresponding Universal Probe, 2  $\mu$ l LightCycler TaqMan Master, and 6.5  $\mu$ l H<sub>2</sub>O. Thermocycling was carried out as follows: initial denaturation was performed at 95°C for 10 min, whereas amplification was achieved by 45 cycles at 95°C for 10 s, at 60°C for 30 s, and at 72°C for 1 s. Finally, a 10-s cooling step to 40°C was performed. Absolute quantification of the Fzd transcripts was performed using a standard curve with different Fzd plasmid dilutions that contained respective Fzd copy numbers. This strategy allowed the absolute quantification of each Fzd. Water instead of cDNA was used as a negative control.

Fzd-specific primers were designed using the Assay Design Center software from Roche (<https://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp>). The primer sequences were as follows:

Fzd1

Forward: 5'-GAACTCCACAAACCTTCCAAA-3'

Reverse: 5'-CGAGCAAGGAGGAATTGTA-3'

Fzd2

Forward: 5'-CCATCCTATCTCAGCTACAAGTTTCT-3'

Reverse: 5'-GCAGCCCTCCTTCTTGGTG-3'

Fzd3

Forward: 5'-GGCTATGGTGGATGATCAAAG-3'

Reverse: 5'-TAACTGCAGGGCGGTGACCT-3'

Fzd4

Forward: 5'-ACACCGCTCATCCAGTACG-3'

Reverse: 5'-TGCACATTGGCACATAAACA-3'

Fzd5

Forward: 5'-CCTGGAGGTGCACCAAGT-3'

Reverse: 5'-TGGGCGGTGTACATAGAGCAT-3'

Fzd6

Forward: 5'-GAAGCAAAAAGACATGCACAGA-3'

Reverse: 5'-TTCGACTTCACTGATTGGATCT-3'

Fzd7

Forward: 5'-AAGCGGTTTGGATGAAAAGA-3'

Reverse: 5'-GATTCACATCGCCGTTATCA-3'

Fzd8

Forward: 5'-CGCCACGCGTTAATTTCT-3'

Reverse: 5'-ATCTCGGGTTCTGGAAACG-3'

Fzd9  
Forward: 5'-GGTCTGACGCTCACCTG-3'  
Reverse: 5'-AGGCAGCCATGTGGAAATAG-3'

Fzd10  
Forward: 5'-TTGGTTTTCACGCAAGG-3'  
Reverse: 5'-CCACAAATTAGTTACACAAGAGGCTA-3'

For quantification of APC,  $\beta$ -catenin, and GAPDH, the LightCycler-FastStart DNA Master SYBR Green I Kit (Roche) was used. Primer sets for the absolute quantification of APC,  $\beta$ -catenin, and GAPDH were designed by Search LC (Heidelberg, Germany). PCR was performed with 1  $\mu$ l cDNA as a template, 1  $\mu$ l primer set (Search LC), 1  $\mu$ l LC-FastStart DNA Master SYBR Green I mix (Roche), and 3  $\mu$ l H<sub>2</sub>O. The conditions for thermocycling were as follows: initial denaturation was carried out at 95°C for 10 min and amplification was achieved by 45 cycles at 95°C for 10 s, at 68°C for 10 s, and at 72°C for 16 s. The exact quantification of mRNA equivalents was carried out by comparing the number of cycles to a given standard with absolute copy numbers (Search LC). The specificity of the primers and lack of secondary amplification was assured by analyzing each PCR by melting curve analysis combined with agarose gel electrophoresis. Water instead of cDNA was used as a negative control.

## siRNA transfection of hMSCs

siRNAs were designed according to the protocol of Reynolds et al. (2004). Sense and antisense oligonucleotides were synthesized by Qiagen. The siRNA sequences were as follows:

| siRNA                 | Gene bank ID | Target sequence                   |
|-----------------------|--------------|-----------------------------------|
|                       |              | Sense sequence                    |
|                       |              | Antisense sequence                |
| Negative control (nc) | -            | 5'-r(UUCUCCGAACGUGUCACGU)d(TT)-3' |
| APC                   | NM_000038    | 5'-r(ACGUGACACGUUCGGAGAA)d(TT)-3' |
|                       |              | 5'-AATGAGAGCACTGATGATAAA-3'       |
|                       |              | 5'-r(UGAGAGCACUGAUGAUAAA)d(TT)-3' |
| $\beta$ -Catenin      | NM_001904    | 5'-r(UUUUAUCAUCAGUCUCUCA)d(TT)-3' |
|                       |              | 5'-AATGGTTGCCTTGCTCAACAA-3'       |
|                       |              | 5'-r(UGGUUGCCUUGCUCAACAA)d(TT)-3' |
|                       |              | 5'-r(UUGUUGAGCAAGGCAACCA)d(TT)-3' |
| Fzd1                  | NM_003505    | 5'-AAGCACGACATCGCTACAA-3'         |
|                       |              | 5'-r(GCACGGACAUCGCGUACAA)d(TT)-3' |
|                       |              | 5'-r(UUGUACGCGAUGUCCGUGC)d(TT)-3' |
| Fzd2                  | NM_001466    | 5'-r(AACGGTCTACATGATCAATA)-3'     |
|                       |              | 5'-r(CGGUCUACAUGAUGAAUA)d(TT)-3'  |
|                       |              | 5'-r(UAUUUGAUGAUGUAGACCG)d(TT)-3' |
| Fzd3                  | NM_017412    | 5'-r(AATGCCAAGATTGCTTATA)-3'      |
|                       |              | 5'-r(UGCCAAGAUUUGCCUUAUA)d(TT)-3' |
|                       |              | 5'-r(UAUUAGGCAAAUCUUGGCA)d(TT)-3' |
| Fzd4                  | NM_012193    | 5'-r(AACATTGTCTCTTGATTAT)-3'      |
|                       |              | 5'-r(CCAUUGUCAUCUUGAUUAU)d(TT)-3' |
|                       |              | 5'-r(AUAAUCAAGAUACAAGG)d(TT)-3'   |
| Fzd5                  | NM_003468    | 5'-r(AATCTCTGCTGATGATTACA)-3'     |
|                       |              | 5'-r(UCCUCUGCAUGGAUUAUAA)d(TT)-3' |
|                       |              | 5'-r(UUGUAAUCCAUGCAGAGGA)d(TT)-3' |
| Fzd6                  | NM_003506    | 5'-r(AACAAGAACTTTGACAGAAA)-3'     |
|                       |              | 5'-r(CAAGAAACUUUGACAGAAA)d(TT)-3' |
|                       |              | 5'-r(UUUCUGUCAAGUUUCUUG)d(TT)-3'  |

|         |           |                                    |
|---------|-----------|------------------------------------|
| Fzd7    | NM_003507 | 5'-r(AAGGAAATGAAGAGGTTTTG)-3'      |
|         |           | 5'-r(GGAAUUGUAAAGAGGUUUUG)d(TT)-3' |
|         |           | 5'-r(CAAAACCUUUAUUAUUUCC)d(TT)-3'  |
| Fzd8 #1 | NM_031866 | 5'-AACTCTGTGCATGGACTACAA-3'        |
|         |           | 5'-r(CUCUGUGCAUGGACUACAA)d(TT)-3'  |
|         |           | 5'-r(UUGUAGUCCAUGCAGAGAG)d(TT)-3'  |
| Fzd8 #2 | NM_031866 | 5'-AAGTACTTCATGTGCCTAGTA-3'        |
|         |           | 5'-r(GUACUUAUGUGCCUAGUA)d(TT)-3'   |
|         |           | 5'-r(UACUAGGCACAUGAAGUAC)d(TT)-3'  |

One day before siRNA transfection, 64 000 hMSCs were plated in a cavity of a six-well plate by adding 2 ml hMSC medium, resulting in 30% confluence after 24-h incubation. For preparing the transfection mixtures, the respective siRNAs were added to 250  $\mu$ l serum-free DMEM in a final concentration of 40 nM. In a separate tube, 8  $\mu$ l Lipofectamine 2000 (Invitrogen, Grand Island, NY, USA) were diluted in 250  $\mu$ l serum-free DMEM. After unifying both solutions, the final transfection mixture was incubated for 20 min at room temperature. This transfection solution was applied to the cells and after an incubation period of 6 h replaced by 2 ml hMSC medium.

## Cloning of pN3-Bar-GLuc

The plasmid pN3-Bar-GLuc was constructed to monitor  $\beta$ -catenin-dependent transcriptional activation of the secreted reporter protein *Gaussia* luciferase. For this purpose, pGLuc-Basic (NEB, Ipswich, MA, USA) was digested with *Hind*III and *Xba*I to isolate the coding sequence for the *Gaussia* luciferase. This fragment was cloned into the plasmid pN3-Mock (pEGFP-N3; Clontech, Mountain View, CA, USA; without the coding sequence of EGFP) through the same restriction sites. Subsequently, the CMV promoter of this vector (pN3-GLuc) was removed by restriction with *Ase*I and *Hind*III (pN3-GLuc $\Delta$ CMV). However, a minimal promoter (P<sub>min</sub>) (CGA-GATCTAGACTCTAGAGGGTATATAATGGAAGCT) carrying the TATA box remained in the pN3-GLuc $\Delta$ CMV-construct. By a PCR approach, the DNA sequence of 12 TCF/LEF binding sites (Bar,  $\beta$ -catenin activated reporter) was amplified with 5'-elongated primers carrying the restriction sites *Ase*I and *Hind*III, respectively (forward primer: AATTAATAATCGATAGATCAAAGGGGG; reverse primer: TTAATAA-GCTTCCAACAGTACCGGAATGC). The plasmid pGL3-Bar (kindly provided by Dr. Randall Moon's laboratory, University of Washington, Seattle, WA, USA) was used as PCR template. After DNA sequencing, the *Ase*I/*Hind*III-digested PCR product was cloned into the *Ase*I/*Hind*III-restricted pN3-GLuc $\Delta$ CMV. The final TCF/LEF reporter gene plasmid was then termed pN3-Bar-GLuc. The respective control plasmid pN3-fuBar-GLuc (found unresponsive Bar) was generated by replacing the original 12 TCF/LEF consensus sequences by 12 mutated TCF/LEF binding sites.

## Determination of the transcriptional start sites of Fzd8

The transcriptional start sites of Fzd8 were determined by 5'-RLM-RACE (Invitrogen) with 250 ng mRNA from hMSCs. 5'-RLM-RACE ensures that only full-length mRNA molecules are selectively reverse



transcribed and amplified by nested PCR. The following Fzd8-specific primers were used: outer reverse, 5'-tcttgctgtcgtggtgaactgattgg-3'; inner reverse, 5'-tgcccttacacagcgacggtgatctc-3'. The PCR products were cloned into the plasmid pCR4-TOPO (Invitrogen), and the DNA sequence of the inserts were determined by a commercial service (Se-quiserve, Vaterstetten, Germany).

## Generation of TCF/LEF-reporter-hMSCs

hMSCs ( $3.8 \times 10^5$ ) were seeded in a 10-cm dish (Nunc, Rochester, NY, USA) to a final confluence of 30%. Transfection of pN3-Bar-GLuc was performed according to our established transfection protocol of plasmid DNA (Hoelters et al., 2005). Briefly, 0.2  $\mu$ g plasmid DNA per square centimeter was used for the hMSC transfections. Accordingly, 15.7  $\mu$ g of pN3-Bar-GLuc were diluted with a 1.5-fold amount of Lipofectamine 2000 in a final volume of 2 ml serum-free DMEM. For selection of stably transfected hMSCs, cells were cultivated in the presence of the antibiotic G418 (100  $\mu$ g/ml). Stably transfected hMSCs carrying the plasmid pN3-Bar-GLuc were named TCF/LEF-reporter-hMSCs.

## Gaussia luciferase assay

For measuring differences in *Gaussia* luciferase activity as a consequence of  $\beta$ -catenin translocation into the nucleus, 12 500 TCF/LEF-reporter-hMSCs were plated into the cavities of 24-well plates 1 day before stimulation with Wnt3a or siRNA transfection. At intervals of 1, 3, and 7 days, 25  $\mu$ l of the supernatant were transferred into the cavities of a black 96-well plate to measure luciferase activity (Costar, New York, NY, USA). Owing to an intrinsic signal peptide of this special luciferase variant, reporter gene activity was measured in cell culture supernatants by using the *Gaussia* luciferase assay kit (PJK, Kleinblittersdorf, Germany) according to the manufacturer's protocol. Immediately after adding 50  $\mu$ l of the substrate, luminescence was quantified in a bioluminescence reader (Tecan, Männedorf, Switzerland).

## Cloning of Fzd1, Fzd5, Fzd7, and Fzd8

The coding sequences of Fzd1, Fzd5, Fzd7, and Fzd8 were amplified using Phusion Hot Start High-Fidelity DNA Polymerase (Finnzymes, Vantaa, Finland) with 5'-elongated primers, using pCMV6-XL4-Fzd1, pCMV6-XL4-Fzd5, pOTB4-Fzd7 (all from Origene, Rockville, MD, USA), or hMSC-cDNA as templates. The full-length coding sequences of the respective Fzds were inserted into pN3-Mock (pEGFP-N3, Clontech, without the coding sequence of EGFP). To facilitate detection of ectopically expressed Fzd on protein level, C-terminal HA-tag sequences were introduced. The primer sequences were as follows:

Fzd1

Forward: 5'-AAGCTTATGGCTGAGGAGGA-3'

Reverse: 5'-AGGGGAGACTACAGTCTGAACTAGT-3'

Fzd5

Forward: 5'-AAGCTTATGGCTCGGCCTGACCCATC-3'

Reverse: 5'-ACTAGTCTACACGTGCCACAGGACAC-3'

Fzd7

Forward: 5'-CCGGAATTCATGCGGGACCCCGGC-3'

Reverse: 5'-CGGGGTACCTCATACCGCAGTCTCCCC-3'

Fzd8

Forward: 5'-GCATCGATATGTTTCAGCCAGGACGAGCGGCC-3'

Forward ( $\Delta$ 52): 5'-GCGCTAGCATGCCCAATCAGTTCAACCACG-3'

Reverse: 5'-GCGATATCTCAGACCTGGGACAATGGCATC-3'

## Transient transfection and co-transfection of hMSCs

A total of 64 000 hMSCs were seeded in a cavity of a six-well plate 1 day before transfection. Transient transfection with Lipofectamine 2000 was carried out according to the protocol of the supplier (Invitrogen). Three micrograms of plasmid DNA per well and 5.25  $\mu$ l Lipofectamine 2000 per well were separately diluted in 250  $\mu$ l serum-free DMEM (PAA). For co-transfection with the TCF/LEF reporter gene plasmid, hMSCs were transfected with 0.6  $\mu$ g pN3-Bar-GLuc per well and 2.4  $\mu$ g of the plasmid of interest per well. After a 5-min incubation step, the solutions were pooled and the transfection mixture was incubated for 20 min at room temperature. hMSCs were transfected by a dropwise application of the mixture. Finally, the transfection solution was replaced by 2 ml  $\alpha$ MEM 6 h after the transfection procedure.

## Western blotting

Upon transient transfection with the Fzd overexpression vectors, cells were cultivated for 2 days and then harvested for protein isolation. Cells were washed with ice-cold 1 $\times$  PBS (PAA) and incubated with 60  $\mu$ l lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% NP40, 1% Triton X-100, 0.1% SDS, 5 mM iodoacetamide, 1:1000 EDTA-free Protease Inhibitor Cocktail Set III; Calbiochem, Darmstadt, Germany) for 10 min on ice. After scrapping off on ice, an incubation of 30 min on ice was carried out. Finally, the samples were centrifuged (16 100 $\times$ g, 5 min, 4°C) and 5 $\times$  DTT loading buffer (0.25 M Tris-HCl, pH 6.8, 0.35 M SDS, 50% glycerol, 0.5 M DTT, 1.45 mM bromophenol blue Na-salt) was added to the sample supernatants. Subsequently, the samples were incubated for 5 min at 95°C, electrophoretically separated using SDS-PAGE, and blotted onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). Membrane blocking was carried out in a 5% milk powder solution at 4°C overnight. After washing with 1 $\times$  TBS-T (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20), membranes were incubated for 1 h at room temperature with a primary anti-HA HRP-labeled antibody (anti-HA-Peroxidase, High Affinity; clone 3F10, Roche), which was diluted 1:2000 in 1 $\times$  TBS-T. After 3 $\times$  10-min washing with 1 $\times$  TBS-T, visualization of protein bands was performed using Western-Lightning Plus-ECL according to the manufacturer's protocol (Perkin Elmer, Waltham, MA, USA). For the detection of the housekeeping gene *GAPDH*, the identical volumes of the same cell lysates (see above) were used. For *GAPDH* protein detection, a polyclonal primary mouse antibody against *GAPDH* (Calbiochem; diluted 1:10 000 in 1 $\times$  TBS-T) was applied for 1 h. After three washing steps with 1 $\times$  TBS-T (3 $\times$  10 min), the respective HRP-conjugated goat anti-mouse secondary antibody (Calbiochem; diluted 1:10 000 in 1 $\times$  TBS-T) was applied for 1 h. After 3 $\times$  10 min washing with 1 $\times$  TBS-T, visualization of protein bands was performed using Western-Lightning Plus-ECL according to the manufacturer's protocol (Perkin Elmer).



## Statistical analysis

Statistical significance was assessed by comparing original values with Student's *t*-test for independent groups. Significance was assumed for \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$ . Statistical analysis was performed using SigmaPlot 10.0 (Systat Software Inc., USA).

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