A novel role for neural cell adhesion molecule in modulating insulin signaling and adipocyte differentiation of mouse mesenchymal stem cells

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Summary

Neural cell adhesion molecule (NCAM) has recently been found on adult stem cells, but its biological significance remains largely unknown. In this study, we used bone-marrow-derived mesenchymal stem cells (MSCs) from wild-type and NCAM knockout mice to investigate the role of NCAM in adipocyte differentiation. It was demonstrated that NCAM isoforms 180 and 140 are expressed on almost all wild-type MSCs. Upon adipogenic induction, Ncam–/– MSCs exhibited a marked decrease in adipocyte differentiation compared with wild-type cells. The role of NCAM in adipocyte differentiation was also confirmed in NCAM-silenced preadipocyte 3T3-L1 cells, which also had a phenotype with reduced adipogenic potential. In addition, we found that Ncam–/– MSCs appeared to be insulin resistant, as shown by their impaired insulin signaling cascade, such as the activation of the insulin–IGF-1 receptor, PI3K–Akt and CREB pathways. The PI3K–Akt inhibitor, LY294002, completely blocked adipocyte differentiation of MSCs, unveiling that the reduced adipogenic potential of Ncam–/– MSCs is due to insulin resistance as a result of loss of NCAM function. Furthermore, insulin resistance of Ncam–/– MSCs was shown to be associated with induction of tumor necrosis factor α (TNF-α), a key mediator of insulin resistance. Finally, we demonstrated that re-expression of NCAM-180, but not NCAM-120, inhibits induction of TNF-α and thereby improves insulin resistance and adipogenic potential of Ncam–/– MSCs. Our results suggest a novel role of NCAM in promoting insulin signaling and adipocyte differentiation of adult stem cells. These findings raise the possibility of using NCAM intervention to improve insulin resistance.

Key words: NCAM, Insulin signaling, Adipocyte differentiation, PI3K–Akt, TNF-α

Introduction

The neural cell adhesion molecule (NCAM), also known as CD56, belongs to the immunoglobulin superfamily (Jorgensen and Bock, 1974; Rutishauser et al., 1976). Widely expressed on neural cells of the central and peripheral nervous system (Schmid et al., 1999), NCAM has been implicated in neurite outgrowth, cell–cell adhesion, synaptic plasticity, learning and memory (Angata et al., 2004; Eckhardt et al., 2000; Weinhold et al., 2005). NCAM is also expressed on other cell types such as epithelial cells, natural killer cells, skeletal muscle and pancreatic β-cells (Cavallaro and Christofori, 2004). There are many different NCAM isoforms that are encoded by a single gene, NCAM1, via mRNA alternative splicing of a number of exons in this gene (Hansen et al., 2008). The three major isoforms of NCAM are NCAM-120, NCAM-140 and NCAM-180, each named according to their apparent molecular masses. These three isoforms share similar extracellular domain, but NCAM-120 lacks a transmembrane domain and is linked to the membrane via a glycosylphosphatidylinositol anchor (Cavallaro and Christofori, 2004). NCAM-140 and NCAM-180 have a transmembrane domain and cytoplasmic tails of different lengths. Compared with NCAM-140, NCAM-180 has an additional 268 amino acid sequence. Generally, the NCAM-120 isoform is expressed in normal and well differentiated tissues, and the other two isoforms are found predominantly in less differentiated cell types (Jensen and Berthold, 2007).

The function of NCAM in the nervous system has been well illustrated (Kiss and Muller, 2001). Upon homophilic or heterophilic binding between cell and cell or cell and extracellular matrix, NCAM is able to activate a complex network of intracellular signaling cascades. It has been well documented that in neurite outgrowth, NCAM can activate fibroblast growth factor receptor (FGFR)-dependent or FGFR-independent signaling molecules, including Fyn, FAK, GAP-43, PLCγ–PKC and PKA (Beggs et al., 1997; Francavilla et al., 2009; Kolkova et al., 2000). Therefore, NCAM is sometimes regarded as a functional receptor (such as a receptor for glial cell line-derived neurotrophic factor), rather than only a mechanical cell adhesion molecule (Jensen and Berthold, 2007; Paratcha et al., 2007). However, it is noteworthy that NCAM does not possess any known intracellular catalytic activity although it can initiate many intracellular signaling pathways (Ditlevsen et al., 2008).

Recently, NCAM was also found to be expressed on mesenchymal stem cells (MSCs) (Bühring et al., 2009; Crigler et al., 2006), but its significance is largely unknown. MSCs isolated from mice and monkeys were shown to express NCAM, which was speculated to contribute to the hematopoietic system (Chu et al., 2006; Wang et al., 2005; Wang et al., 2010). After two to three weeks in culture, the expression of NCAM on MSCs was drastically increased compared with freshly isolated bone marrow cells (Kato et al., 2008). MSCs are adult stem cells with multipotency defined by their ability to differentiate into adipocytes, osteoblasts,
chondrocytes, myocytes and even neural cells (Nagai et al., 2007; Pittenger et al., 1999; Yang et al., 2008). Thus, it would be interesting to explore the possible role of NCAM in the differentiation potential of MSCs, when considering the multipotent nature of MSCs and various functions of NCAM.

In the study reported here, we demonstrated that the adipocyte differentiation potential was impaired in both Ncam−/− MSCs and NCAM-silenced preadipocyte 3T3-L1 cells in vitro, in addition to reduced insulin signaling. Further investigation revealed that NCAM deficiency induces tumor necrosis factor α (TNF-α) expression and insulin resistance.

Results
NCAM deficiency impairs adipocyte differentiation of mouse MSCs in vitro

It is well known that MSCs possess multipotency, defined by the ability to differentiate into adipocytes, osteoblasts and chondrocytes (Nagai et al., 2007). To explore the role of NCAM in the adipocyte differentiation of MSCs, we isolated bone-marrow-derived MSCs from wild-type and Ncam−/− (knockout; KO) mice. FACS analysis of wild-type MSCs demonstrated that more than 99.1% of the cells were NCAM positive (Fig. 1A), suggesting that almost all wild-type MSCs are NCAM-expressing cells. Western blot analysis demonstrated that only NCAM-140 and NCAM-180 were expressed in wild-type MSCs, whereas NCAM-120 expression was not detected under our experimental conditions. As a negative control, none of NCAM isoforms was detected on Ncam−/− MSCs (Fig. 1B).

To determine the effect of NCAM deficiency on adipocyte differentiation of MSCs, wild-type and Ncam−/− MSCs were treated with MDI adipogenic differentiation medium containing insulin, 3-isobutyl-1-methylxanthine (IBMX) and dexamethasone for 3 days, and further cultured in the medium containing insulin alone. After a 14-day treatment, the adipogenic phenotype was assessed by Oil-Red-O staining. As shown in Fig. 1C,D, the lipid accumulation was significantly less in the Ncam−/− MSCs, as compared with wild-type ones. Moreover, quantitative analysis of intracellular lipid droplets demonstrated that after a 14-day adipogenic induction, lipid content of Ncam−/− MSCs was only 30±4.6% that of wild-type MSCs (Fig. 1E), indicating that the loss of NCAM severely impairs the adipogenic capacity of MSCs.

To further characterize the defect of fat accumulation in Ncam−/− MSCs, we examined the markers of adipocyte differentiation by western blotting. As shown in Fig. 1F, peroxisome proliferator-activated receptor (PPARγ) in wild-type MSCs was considerably upregulated during adipocyte differentiation. By contrast, the induction of PPARγ in Ncam−/− MSCs was significantly lower than that in wild-type cells. The protein expression pattern of another adipocyte marker, fatty acid-binding protein (αP2), was similar to that of PPARγ. At day 3 post-induction, αP2 in wild-type cells was strikingly induced, whereas no obvious αP2 induction was observed in KO cells, further supporting the conclusion that adipocyte differentiation of Ncam−/− MSCs is impaired. It is noteworthy that during the whole differentiation process, the expression level of NCAM-140 and NCAM-180 in wild-type cells remained unchanged; however, no NCAM-120 induction was observed during adipocyte differentiation of wild-type cells.

NCAM silencing inhibits differentiation of preadipocyte 3T3-L1 cells

To further confirm the role of NCAM in adipocyte differentiation, we chose the well-established preadipocyte cell line 3T3-L1 as an alternative in vitro model for differentiation study. Western blotting showed that NACM-140 and NCAM-180 were clearly expressed but no NCAM-120 expression was detected in 3T3-L1 cells (data not shown), as in MSCs. Next, plasmid-based small interfering RNA (siRNA) silencing of gene expression was utilized to develop mixed cell lines with stable NCAM downregulation. As shown in Fig. 2A, NCAM siRNA-transfected cells had a markedly lower expression of NCAM-140 and NCAM-180 compared with the control siRNA-transfected cells. Both cell populations were

![Fig. 1. NCAM deficiency impairs adipocyte differentiation of mouse MSCs in vitro.](image-url)
incubated with MDI for 3 days and cultured for another 11 days in the presence of insulin, and then the intracellular lipid accumulation was visualized with Oil-Red-O staining. After an induction of 14 days, almost all control siRNA-transfected 3T3-L1 cells became lipid droplet-containing adipocytes, whereas only just under than half of NCAM siRNA-transfected cells contained lipid droplets (Fig. 2B). As negative controls, non-induced cells from both cell lines showed no significant fat staining. Consistently, the quantitative analysis of intracellular lipid droplets demonstrated that after a 14-day adipogenic induction, the lipid content of NCAM siRNA-transfected cells was only 45.9±3.3% of that in wild-type 3T3-L1 cells (Fig. 2C).

Also, western blotting indicated that upon adipogenic induction for 3 or 7 days, NCAM-silenced 3T3-L1 cells expressed less PPARγ and αP2 than control siRNA-transfected 3T3-L1 cells (Fig. 2D). Taken together, these results further confirmed that the NCAM level correlates with the adipogenic differentiation potential of 3T3-L1 cells, suggesting that NCAM might play an important role in adipogenesis of various cell lines.

**Wnt signaling does not contribute to NCAM deficiency-impaired adipocyte differentiation**

Wnts are a family of secreted glycoproteins that regulate cell growth and cell fate (Logan and Nusse, 2004). There is a considerable body of evidence suggesting that Wnts are key negative regulators of adipocyte differentiation in vitro (Rosen and MacDougald, 2006). Conversely, inhibiting the Wnt pathway alone can result in spontaneous adipogenesis of mesenchymal precursors (Rosen et al., 2000). Considering the crucial role of Wnt signaling in adipogenesis, we determined the effect of NCAM on Wnt signaling during adipocyte differentiation of MSCs (Ross et al., 2000; MacDougald, 2006). Conversely, inhibiting the Wnt pathway alone can result in spontaneous adipogenesis of mesenchymal precursors (Rosen et al., 2000; MacDougald, 2006). Therefore, these findings might rule out the possibility that the effect of NCAM on adipocyte differentiation of MSCs is mediated by Wnt signaling.

**PI3K–Akt signaling contributes to NCAM-mediated adipogenic differentiation**

It has been shown that the PI3K–Akt (phosphoinositide 3-kinase–protein kinase B) signaling cascade plays a crucial role in adipocyte differentiation of MSCs (Ross et al., 2000; Garofalo et al., 2003). To understand the mechanism for the impaired adipocyte differentiation of Ncam−/− MSCs, we examined the effect of NCAM deficiency on the activation of PI3K–Akt signaling. Cells were treated with MDI for 0, 10, 30 and 120

![Fig. 2. NCAM silencing inhibits differentiation of preadipocyte 3T3-L1 cells. (A) 3T3-L1 cells were transfected with pSilencer-4.1-based plasmid containing a scrambled sequence (control; Si-ctl) or a 19-bp insert targeting mouse NCAM (Si-NCAM), and analyzed by immunoblotting with anti-NCAM antibodies. β-actin was detected as a loading control. (B) Cells transfected with control or NCAM siRNA were induced with MDI medium. The cells were stained with Oil Red O at day 14. (C) The lipid content of differentiated cells was quantified by extraction and measurement of the absorption at 490 nm. *P<0.01, compared with differentiated control siRNA-transfected cells. (D) Cells were treated with MDI medium for 0, 3 and 7 days and adipocyte markers PPARγ and αP2 were analyzed by immunoblotting.

![Fig. 3. Wnt signaling does not contribute to NCAM deficiency-impaired adipocyte differentiation. (A) Wild-type MSCs were pretreated with the Wnt agonist LiCl (25 mM) for 1 hour before MDI induction for 7 days. Adipocyte differentiation markers PPARγ and αP2 were analyzed by immunoblotting. β-actin was detected as a loading control. (B) Wild-type MSCs were treated with LiCl (25 mM) for 1 hour before MDI induction. The cells were fixed and stained with Oil Red O at day 14. (C) Wild-type (WT) and Ncam−/− (KO) MSCs were treated with MDI medium for 0, 3 and 7 days and the expression levels of β-catenin were analyzed by immunoblotting.
minutes, and proteins were extracted to detect the activation of PI3K–Akt signaling. Compared with wild-type MSCs, knockout of NCAM led to a sharp decrease in Akt phosphorylation, which was most evident at 30 and 120 minutes after MDI stimulation (Fig. 4A). Consistently, cAMP response element–binding protein (CREB), a downstream molecule of the Akt pathway was also impaired in KO cells, as shown by the attenuated phosphorylation of CREB.

To determine if the NCAM-mediated activation of Akt is involved in adipocyte differentiation, we applied the Akt inhibitor LY294002 (LY) during adipogenic induction. As shown in Fig. 4B, phosphorylation of Akt was inhibited by LY in a dose-dependent manner, and completely abolished when the concentration of LY increased to 10 μM. After 7 days of adipogenic induction, LY at 10 μM totally inhibited the induction of PPARγ and α2 in MSCs (Fig. 4C). Accordingly, lipid accumulation and adipocyte differentiation were also completely blocked by LY (Fig. 4D,E), indicating a crucial role of NCAM-mediated PI3K–Akt signaling in adipocyte differentiation of MSCs.

NCAM-180 restores PI3K–Akt signaling and adipocyte differentiation of Ncam−/− MSCs

As mentioned above, loss of NCAM function impaired both the adipocyte differentiation capacity and PI3K–Akt signaling of MSCs. To determine which NCAM isoforms are responsible for such impairments, Ncam−/− MSCs were transfected with plasmids expressing full-length mouse NCAM-140 and NCAM-180, respectively. Upon short-term selection with neomycin, mixed stable cell lines were obtained. As shown in Fig. 5A, the levels of NCAM-140 and NCAM-180 expression in the transfected KO cells were comparable with those seen in wild-type cells. Upon MDI stimulation, transfected KO cells expressing either NCAM-140 or NCAM-180 showed significantly increased phosphorylation of Akt, compared with the untransfected KO cells (Fig. 5B). However, NCAM-180 was more effective at restoring PI-3K–Akt signaling than NCAM-140, suggesting that NCAM-180, but not NCAM-140, plays a predominant role in enhancing the efficiency of PI3K–Akt signaling.

When subjected to adipogenic induction, transfected KO cells expressing MCAM-180 showed an increase in adipogenic markers, such as PPARγ and α2, compared with control KO cells (Fig. 5C). Consistent with this observation, the quantitative determination of intracellular lipid droplets showed that NCAM-180 expression could also restore the accumulation of fat in KO cells (Fig. 5D). By contrast, expression of NCAM-140 in the KO cells contributed less to the induction of adipogenic markers as well as fat accumulation, supporting the pivotal role of NCAM-180 in the adipocyte differentiation of MSCs.

NCAM deficiency reduces activation of insulin receptor

The above data suggest that the loss of NCAM in MSCs impairs PI3K–Akt signaling upon MDI mixture treatment. To determine the underlying mechanism, we analyzed the effects of the individual component of the MDI mixture on PI3K–Akt signaling. Wild-type MSCs were treated with insulin, IBMX, dexamethasone (DMS) or MDI mixture for 30 minutes. As shown in Fig. 6A, only insulin and MDI mixture were able to induce phosphorylation of Akt, whereas both IBMX and DMS could not, indicating that the insulin in the MDI mixture is the crucial agent for PI3K–Akt activation. Therefore, we chose insulin as an inducer in the following experiments.

To understand how the insulin-induced PI3K–Akt signaling was impaired in Ncam−/− MSCs, we next examined the phosphorylation of insulin receptor (IR), which is the first step in the activation of the insulin signaling cascade. As shown in Fig. 6B, the expression level of IR in wild-type and Ncam−/− MSCs was identical. However, when subjected to insulin treatment, the tyrosine phosphorylation of IR in KO cells was greatly suppressed, as compared with wild-type cells. This indicates that NCAM deficiency can impair insulin resistance at the level of IR.

To further confirm the impaired activation of IR in KO cells, we examined the level of total tyrosine autophosphorylation of IR by immunoprecipitation (IP). As expected, we observed a significant decrease in insulin-stimulated IR tyrosine autophosphorylation in Ncam−/− cells compared with that in wild-type cells (Fig. 6C), reinforcing the conclusion that NCAM deficiency can impair insulin signaling at the IR level.

Again, to test which NCAM isoforms were responsible for the impaired activation of IR, we examined the effect of induced expression of NCAM-140 and NCAM-180 on activation of IR. Upon insulin treatment, restored tyrosine phosphorylation of IR was only observed in transfected KO cells expressing NCAM-180 (Fig. 6D). By contrast, NCAM-140 transfection did not rescue the decreased phosphorylation of IR in KO cells, revealing that NCAM-180 is more important than NCAM-140 in facilitating the insulin response of MSCs.
NCAM deficiency induces insulin resistance via TNF-α induction

TNF-α is an important mediator of insulin resistance through its ability to decrease the tyrosine kinase activity of the IR (Hotamisligil et al., 1993; Sarac et al., 2010). To determine whether the expression level of TNF-α is different in non-stimulated wild-type and Ncam−/− MSCs, real-time RT-PCR was performed. As shown in Fig. 7A, the expression of TNF-α mRNA in KO cells increased to 4.3-fold that in wild-type MSCs. When reactivated with NCAM-180, the expression of Tnfa mRNA in KO cells was decreased by 59±8.3%, suggesting that the presence of NCAM-180 might inhibit the expression of TNF-α, whereas the loss of NCAM might lead to induction of TNF-α. In addition, we examined the amount of TNF-α secreted into the cell culture medium, using ELISA. As shown in supplementary material Fig. S1, 10.2±5.5, 62.0±15.1 and 26.3±6.7 pg/ml of TNF-α were detected in the culture medium of three cell types (wild type, KO and KO transfected, respectively). The results were consistent with qPCR data and further confirmed the regulation of NCAM on TNF-α expression.

It has been shown that TNF-α induces serine phosphorylation of insulin receptor substrate-1 (IRS-1) and thereafter converts IRS-1 into an inhibitor of the IR tyrosine kinase activity in vitro (Hotamisligil et al., 1996; de Alvaro et al., 2004). Thus, we examined the serine phosphorylation levels of IRS-1 in non-stimulated wild-type and Ncam−/− MSCs. As shown in Fig. 7B, KO cells had a substantially increased level of IRS-1 serine phosphorylation compared with wild-type cells. Because IRS-1 serine phosphorylation mediates the inhibition of IR, increased serine phosphorylation of IRS-1 in KO cells might partially explain the impaired insulin signaling in these cells.

Lastly, to determine whether the impaired insulin signaling in KO cells is related to TNF-α, we examined the effect of recombinant TNF-α on the phosphorylation of IR and Akt in wild-type MSCs. As shown in Fig. 7C, in the presence of TNF-α, the basal levels of IR and Akt phosphorylation remained unchanged. By contrast, insulin-induced phosphorylation of IR and Akt was strongly inhibited by recombinant TNF-α, indicating that upregulated TNF-α is at least in part responsible for impaired insulin signaling in Ncam−/− MSCs.

Discussion

NCAM is known to regulate cell–cell adhesion, neurite outgrowth and synaptic plasticity in the nervous system (Angata et al., 2004; Eckhardt et al., 2000; Maness and Schachner, 2007; Weinhold et al., 2005). However, its ubiquitous expression in various cells suggested its diverse functions in different systems. NCAM was found to play a role in cell growth (Francavilla et al., 2009), tumor malignancy (Jensen and Berthold, 2007) and cell type segregation in pancreatic islets (Esnì et al., 1999). The recent findings that adult stem cells and progenitor cells also express NCAM suggest its previously unknown function in stem cells (Evseenko et al., 2010; Kato et al., 2008; Wang et al., 2010).

In the present study, we revealed a novel role of NCAM in mediating adipogenesis of MSCs in vitro. To the best of our knowledge, it is the first investigation to demonstrate the role of NCAM in adipocyte differentiation. Adipogenesis from stem cells consists of two phases. The first phase is differentiation, which converts stem cells into preadipocytes. The second one is terminal differentiation, in which the preadipocytes become mature adipocytes (Rosen and MacDougald, 2006). A previous study suggested that an adipocyte adhesion molecule (ACAM), a 45 kDa protein expressed on adipocytes is involved in adipocyte maturation (Eguchi et al., 2005). In our study, we showed that the adipocyte differentiation in Ncam−/− MSCs is impaired because there was...
NCAM deficiency is always accompanied by the dysregulation of CREB-mediated signaling pathways (Aonurm-Helm et al., 2008). These findings indicate a possible mechanism by which NCAM contributes to insulin signaling pathways, especially in insulin–IGF-1 sensitivity.

Only NCAM-180 and NCAM-140, but not NCAM-120, were found to be expressed on either MSCs or 3T3-L1 cells. Likewise, in the mature adipocytes induced from MSCs or 3T3-L1 cells, NCAM-120 was still not detected, suggesting a unique expression pattern of NCAM isoforms in MSCs and 3T3-L1 cells. It has been suggested that NCAM-140 could activate more signaling molecules whereas NCAM-180 is associated with the cytoskeleton (Walsh et al., 1997). In general, NCAM-140 exhibits a potent role in neutrophil outgrowth. Interestingly, we demonstrated that re-expression of NCAM-180 isoform rather than NCAM-140 rescued the impaired adipocyte differentiation in KO MSCs. Consistently, restoration of NCAM-140 contributed a little to the impaired insulin signaling in KO cells. In contrast, re-expression of NCAM-180 largely restored the reduced insulin signaling in KO cells. These results suggest that NCAM-180 but not NCAM-140 plays a key role in mediating adipogenesis and insulin signaling. Considering that NCAM-180 has just an insertion of 268 amino acids within its intracellular domain compared with NCAM-140, it might indicate that this insertion plays a vital role in mediating insulin sensitivity. Thus, future experiments will be required to identify the mechanism underlying the functional difference of these two isoforms in insulin signaling.

NCAM is also involved in the regulation of some inflammatory factors. NCAM-mimetic peptide FGL can act as an anti-inflammatory peptide. FGL can promote the production of anti-inflammatory cytokine interleukin-4 or inhibit the expression of pro-inflammatory cytokine interferon-γ and interleukin-1β (Downer et al., 2009; Downer et al., 2010). In our study, we found that pro-inflammatory cytokine TNF-α levels were much higher in Ncam−/− than wild-type cells. We also demonstrated that NCAM reconstitution with NCAM-180 can suppress the expression of TNF-α in Ncam−/− MSCs. Furthermore, recombinant TNF-α can induce IR and/or IGFR resistance and thereby PI3K–Akt signaling inhibition, which is consistent with previous results (Hotamisligil et al., 1993; Zhang et al., 2008). TNF-α had been regarded as a negative player in type 2 diabetes (Lele, 2010) and its secretion induces insulin resistance (Hotamisligil et al., 1993; Hotamisligil et al., 1996) via inhibition of IR tyrosine kinase activity. Taken together, these findings reveal an important role of NCAM in the anti-inflammatory response. However, our results indicate that NCAM can promote insulin signaling at least in part via inhibiting the production of TNF-α. A further question is how NACM regulates TNF-α expression. As mentioned above, because the NCAM agonist FGL promotes IGF-1 release and thereby downregulates pro-inflammatory cytokines, we speculate that NCAM does not regulate TNF-α expression in a direct way. Instead, NCAM might stimulate growth factor release and thereby suppress TNF-α expression.

Changes in cell shape are a morphological hallmark of differentiation (Kawaguchi et al., 2003). Generally, upon adipogenic induction, cells will undergo morphological change from a fibroblast-like shape to a spherical one (Gregoire et al., 1998). Without such morphological changes at an early stage, adipocyte differentiation will be repressed. In addition, remodeling of F-actin plays an important role in adipogenesis. In fact, as a cell adhesion molecular, NCAM has been found to play a crucial role in

###Fig. 7. NCAM deficiency induces TNF-α expression.
(A) After re-expression of NCAM-180, relative mRNA levels of TNF-α in Ncam−/− (KO) MSCs were determined by real-time PCR. Data are representative of three independent experiments and values are means ± s.e.m. *P<0.01. (B) Lysates of wild-type (WT) and Ncam−/− MSCs were immunoprecipitated with anti-IRS-1 antibodies or normal mouse IgG (control Ab), and detected with anti-phosphorylated serine (p-Ser) or anti-IRS-1, respectively. (C) Wild-type cells were pretreated with recombinant TNF-α (5 ng/ml) for 1 day, and then treated with insulin (0.1 μg/ml) for 10 minutes. The phosphorylated IR and Akt were analyzed by immunoblotting. (D) Levels of phosphorylated IR and Akt in C were quantified by densitometry and normalized to IR and Akt loading controls, respectively. Data are representative of three independent experiments and values are means ± s.e.m. (bars). *P<0.01 and **P<0.001.

![Graphs showing reduced induction of adipocyte differentiation](image-url)
remodeling of F-actin (Esni et al., 1999; Olofsson et al., 2009). In Ncam−/− mice, the reorganization of the submembrane F-actin network was found to be defective (Olofsson et al., 2009). In pancreatic islets, NCAM is required for islet cell type segregation and the subcellular distribution of F-actin and cadherins (Esni et al., 1999). Interestingly, we did find that Ncam−/− MSCs were less mobile (data to be published), and motility is known to be closely related to F-actin network. Thus, we could not rule out the potential role of NCAM in adipogenesis via remodeling of the F-actin network. Meanwhile, it would be interesting and worthwhile to investigate the contribution of NCAM to cell skeleton changes when stimulated with insulin or other adipogenic induction reagents.

In this work, we demonstrated that NCAM-180 plays a crucial role in mediating the adipocyte differentiation of MSCs. However, in MSCs, adipocyte differentiation still happens upon MDI induction (~30%) in the absence of NCAM-180. One possible reason is that in NCAM KO MSCs, insulin signaling is reduced but not completely abolished. Another reason is that in the absence of NCAM-180, some other adhesion molecules on MSCs might be upregulated to partially compensate for the impaired adipocyte differentiation of MSCs. For example, in mouse models of pancreatic cancer and lobular breast dissemination, NCAM deficiency leads to aberrant persistence of E-cadherin expression (Perl et al., 1999; Derksen et al., 2006).

Taken together, the results of this study reveal a novel role of NCAM (especially the NCAM-180 isoform) in enhancing insulin sensitivity and promoting adipocyte differentiation in both MSCs and preadipocytes. In addition, NCAM deficiency induces TNF-α expression and thereafter leads to insulin resistance and impaired adipocyte differentiation. NCAM agonists such as FGL peptide (Downer et al., 2009) are available currently, thus, the application of agonists in fighting insulin resistance or even type 2 diabetes is expected.

Materials and Methods

Materials

SiRNA expression vectors pSilencer 4.1-CMV neo was purchased from Ambion (Austin, TX). Insulin, dexamethasone and 3-isobutyl-1-methylxanthine (Austin, TX). The mammalian expression vector pcDNA4/Myc was purchased from Invitrogen (Carlsbad, CA). Insulin, dexamethasone and 5 mM PMSF and Roche’s complete protease inhibitors) and centrifuged at 14,000 rpm for 20 minutes at 4°C. The proteins in the supernatant were measured using a Protein Assay Kit II (Bio-Rad, Hercules, CA). For immunoprecipitation analysis, 500 μg of total cell lysates was pre-cleared with protein A plus G-Sepharose before incubation with specific primary antibodies, followed by addition of protein A plus G-Sepharose. The precipitated proteins were resolved in 2X SDS-PAGE sample buffer for electrophoresis.

For western blots, samples were separated by electrophoresis on 9–16% SDS–PAGE, and transferred onto a PVDF membrane (Millipore Corporation, Billerica, MA). After blocking with PBST containing 5% skimmed milk, membranes were incubated with primary antibodies. The membranes were further incubated with horseradish peroxidase-conjugated secondary antibodies and developed using Pierce’s West Pico chemiluminescence substrate. To determine the equivalence of protein amounts loaded of the different samples, the developed membranes were stripped with a buffer consisting of 62.5 mM Tris-HCl (pH 6.7), 2% SDS and 100 mM 2-mercaptoethanol for 1 hour, followed by incubation with control antibodies, such as anti-β-actin, anti-total Akt, anti-CREB and anti-IR, for further blotting. In some cases, immunooblots were quantified by measuring the immunoreactive protein band density with ImageJ 1.43 (NIH) software.

Real-time PCR

Total RNA was extracted using TRIzol reagent following the manufacturer’s instructions (Invitrogen), and reverse transcribed to cDNAs using SuperScript II reverse transcriptase (Invitrogen). mRNA levels were measured using a real-time PCR system (ABI Prism7500, Applied Biosystems, Foster City, CA) and SYBR Green qPCR Master Mix (KAPA Biosystems). The gene-specific primers used for real-time PCR analysis, 5′-AAGTGAAAGCAGCTGGA-3′ and 5′-ACTACAGTTTGGAGGTAA-3′ for NCAM-α, 5′-GCTTTCCTCCCTCTCTCC-3′ and 5′-TGATCACAATCTGCTGAG-3′ for β-actin. The target mRNA level of cell control normalized to the level of β-actin mRNA, was set to 1.

Transfection and gene silencing with siRNA

5×10^5 cells were seeded on 60-mm tissue culture dishes and cultured overnight. Plasmid (8 μg) was transfected into cells using Lipofectamine 2000 at the transfection reagent:DNA ratio of 2 for the transfection reagents following the manufacturer’s instructions. For transient transfection, no selection was needed. To obtain stable cell lines (mixed), cells were selected with neomycin at 800 μg/ml or zeocin at 100 μg/ml for 7–10 days. Gene silencing or re-expression of NCAM was determined by western blotting with anti-NCAM or anti-c-Myc tag antibodies.

FACS analysis of wild-type MSCs

For quantification of NCAM expression on wild-type MSCs, the cells were trypsinized, fixed with 4% parafomaldehyde for 15 minutes, permeabilized with 0.1% saponin for 30 minutes, and then incubated with rabbit polyclonal antibodies against NCAM for 1 hour. Next, the cells were incubated with FITC-conjugated secondary antibodies (Alexa Fluor 488; Invitrogen) for 30 minutes. Lastly, FACS was performed with a FACSCalibur flow cytometer (Becton-Dickinson Corp., Franklin Lakes, NJ).
Statistical analysis

Data are expressed as means ± s.e.m. (standard error of the mean). The group means were compared by ANOVA and significance of differences was determined by post-hoc testing using Bonferroni’s method. A P-value of <0.05 was considered significant.

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