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NG2-mediated Rho activation promotes amoeboid invasiveness of cancer cells

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ABSTRACT

The aim of this study was to analyze the potential role of NG2 chondroitin sulfate proteoglycan in amoeboid morphology and invasiveness of cancer cells. In the highly metastatic amoeboid cell lines A3 and A375M2, siRNA-mediated down-regulation of NG2 induced an amoeboid-mesenchymal transition associated with decreased invasiveness in 3D collagen and inactivation of the GTPase Rho. Conversely, the expression of NG2 in mesenchymal sarcoma K2 cells as well as in A375M2 cells resulted in an enhanced amoeboid phenotype associated with increased invasiveness and elevated Rho-GTP levels. Remarkably, the amoeboid-mesenchymal transition in A375M2 cells triggered by NG2 down-regulation was associated with increased extracellular matrix-degrading ability, although this was not sufficient to compensate for the decreased invasive capability caused by down-regulated Rho/ROCK signaling. Conversely, in K2 cells with overexpression of NG2, the ability to degrade the extracellular matrix was greatly reduced. Taken together, we suggest that NG2-mediated activation of Rho leading to effective amoeboid invasiveness is a possible mechanism through which NG2 could contribute to tumor cell invasion and metastasis.

Introduction

The main cause of mortality by cancer is due to the ability of tumor cells to spread to distant locations in the body and thereby form metastases. The process starts with local invasion to adjacent tissue, which is a key determinant of the metastatic potential. Tumor cells invade either collectively, retaining their intracellular junctions, or individually (reviewed in Panková et al., 2010). Individual cell migration is broadly classified as either mesenchymal or amoeboid (reviewed in Panková et al., 2010; Friedl, 2004; Lämmermann and Sixt, 2009).

Cells with mesenchymal morphology exhibit, in a 3D environment, an elongated spindle-like morphology with one or more leading pseudopodia. Their movement is initiated by the formation of actin rich filopodia and lamellipodia. This process is controlled by the small Rho-GTPases Rac and Cdc42 (Sahai and Marshall, 2003; Sanz-Moreno et al., 2008). To invade the extracellular matrix (ECM), mesenchymal cells in vitro employ specified cellular structures called invadopodia; motile cellular protrusions with an ability to invade the surrounding ECM via its degradation (Caldieri et al., 2009; Tolde et al., 2010).

Cells with amoeboid-like type migration exhibit a round shape in 3D environments. Their movement is characterized by cycles of expansion and contraction of the cell body and bleb like protrusions mediated by cortically localized acto-myosin interactions (Wolf et al., 2003; Sahai and Marshall, 2003). This process is promoted by the up-regulation of Rho/ROCK signaling. The increased Rho/ROCK signaling has been shown to assist in the generation of sufficient acto-myosin forces allowing the amoeboid tumor cells to push through the extracellular matrix independently of the extracellular matrix degradation (Rosel et al., 2008).

Amoeboid and mesenchymal invasion modes are not mutually exclusive. Suppression or enhancement of specific molecular pathways can induce a mesenchymal-amoeboid transition or amoeboid-mesenchymal transition (reviewed in Panková et al., 2010). Invasion by both types of cells requires adhesion to components of the ECM and acto-myosin contraction to promote migration. The adhesion of mesenchymal cells is mainly mediated via integrins; but amoeboid cells typically express integrins at very low levels (Rosel et al., 2008). The low expression of integrins in amoeboid cells is apparently at odds with their increased ability to generate traction forces. It was recently suggested, however, that amoeboid cell adhesion to the ECM can be mediated by non-integrin receptors such as glycoproteins (Friedl, 2004).

NG2 chondroitin sulfate proteoglycan (a.k.a. CSPG-4, MCSP) is an integral membrane proteoglycan found on the surface of many different types of progenitor cells (Nishiyama et al., 1991). NG2

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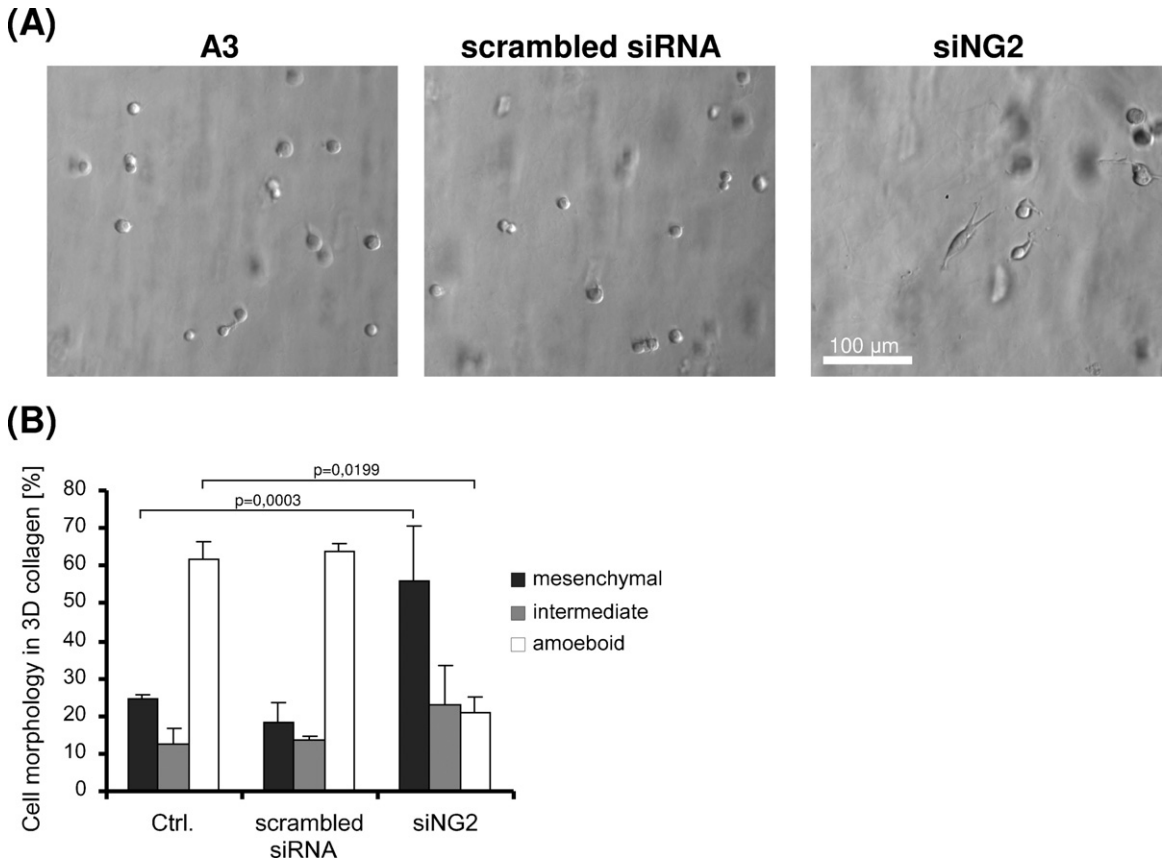

Fig. 1. Effect of NG2 silencing on morphology of A3 sarcoma cells. (A) Cells were grown in 3D collagen and after 24 h cell morphology was analyzed and documented using photomicroscopy. Left panel: A3 cells; medium panel: A3 cells treated with scrambled siRNA; right panel: A3 cells treated with NG2-specific siRNA. (B) Quantitative analysis of the cell morphology. Cells were grown in 3D collagen and after 24 h the morphology of cells was analyzed using photomicroscopy and classified on the basis of the elongation index as described in section "Materials and methods".

was shown to mediate adhesion of cells to collagen and other ECM components (Stallcup et al., 1990; Burg et al., 1996; Tillet et al., 1997). Here we show that NG2 expression has pro-amoeboid effects on tumor cells, whereas its inhibition appears pro-mesenchymal. Our results further suggest that NG2 affects tumor cell behavior through the regulation of Rho. The pro-amoeboid effects of NG2 together with its ability to bind components of ECM suggest that NG2 could be a non-integrin ECM receptor of amoeboid cells.

Materials and methods

Cells and culture

K2 cells, full name LW13K2, are spontaneously transformed rat embryonic fibroblasts. The A3 cells, full name A337/311RP, were developed from K2 by neoplastic progression in vivo and in vitro (Cavanna et al., 2007, PMID: 17264155). K2 and A3 cells were cultivated in MEM with Hanks' salts (HMEM) supplemented with 10% bovine serum (ZVOS), 0.09% sodium bicarbonate, 0.12 g/l sodium pyruvate, and 1 mmol/l glutamine at 37 °C with 5% CO_2. A375M2 melanoma cells (obtained from Prof. R. Hynes) were routinely maintained in DMEM (GIBCO) with 4500 mg/l L-glucose, L-glutamine, and pyruvate, supplemented with 10% fetal bovine serum (Sigma), 2% antibiotic–antimycotic (GIBCO) and 1% MEM non-essential amino acids (GIBCO).

siRNA and cDNA transfection

A375M2, A3 and K2 cells were plated at 60% confluence on 6-well plates and after 24 h transfected with 60 pmol of siRNA using LipofectamineRNAiMax (Life Technologies) for A3 and K2 cells, or jet Prime DNA and siRNA transfection reagent (Polyplus) for A375M2 cells. SiRNAs against human (A375M2) and rat (A3, K2 cells) NG2 were purchased from Life Technologies. For siRNA rescue experiments, the transfection mixture was further supplemented with 5 μg of rat *ng2* cDNA plasmid – kind gift from Prof. W. Stallcup (Sanford-Burnham Medical Research Institute, Cancer Center, La Jolla, CA). Knockdown and reexpression of NG2 was confirmed by flow cytometry. In preliminary testing three siRNA oligos were used. In all cases the successful knockdown of NG2 led to amoeboid-mesenchymal transition. For further experiments only the most effective oligo was chosen.

To achieve stable transfection of K2 cells, the NG2 cDNA was first recloned using BamHI/NotI to pIRESpuro3 vector (Invitrogen) and K2 cells were transfected with the resulting construct. Transfected K2 cells were then selected with 7 μg/ml puromycin for 4 weeks after which time individual colonies were isolated, and NG2 overexpression was confirmed by flow cytometry.

Immunoblotting and Rho pull-down assays

Confluent cell cultures were washed with phosphate buffered saline (PBS) and lysed in modified RIPA buffer (50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% NP40, 1% sodium deoxycholate,