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Involvement of headless myosin X in the motility of immortalized gonadotropin-releasing hormone neuronal cells

Jun-Jie Wang^a, Xiu-Qing Fu^a, Yu-Guang Guo^a, Lin Yuan^a, Qian-Qian Gao^a, Hua-Li Yu^a, Heng-Liang Shi^a, Xing-Zhi Wang^a, Wen-Cheng Xiong^b, and Xiao-Juan Zhu^{a,*}

^aInstitute of Cytology and Genetics, Key Laboratory of Molecular Epigenetics of Ministry of Education, Northeast Normal University, 5268 Renmin Street, Changchun 130024, Jilin, PR China

^bProgram of Developmental Neurobiology, IMMAG and Department of Neurology, Medical College of Georgia, Augusta, GA 30912, USA

Abstract

Myosin X (Myo X), an unconventional myosin with a tail homology 4-band 4.1/ezrin/radixin/moesin (MyTH4-FERM) tail, is expressed ubiquitously in various mammalian tissues. In addition to the full-length Myo X (Myo X FL), a headless form is synthesized in the brain. So far, little is known about the function of this motor-less Myo X. In this study, the role of the headless Myo X was investigated in immortalized gonadotropin-releasing hormone (GnRH) neuronal cells, NLT. NLT cells overexpressing the headless Myo X formed fewer focal adhesions and spread more slowly than the wild-type NLT cells and GFP-expressing NLT cells. In chemotaxis assays, the NLT cells overexpressing the headless Myo X migrated shorter distances and had fewer migratory cells compared with the control NLT cells.

Keywords

Filopodia; Overexpression; Adhesion; Migration

1. Introduction

Myosins are actin-based motors that play a critical role in diverse cellular motile events (Berg et al., 2001; Sellers, 2000). Myosin X (Myo X) is one of the least well-understood unconventional myosins expressed at low concentrations in most vertebrate cells. The heavy chain of full-length Myo X (Myo X FL) can be divided into head, neck and tail (Berg et al., 2000). The head of Myo X is the motor domain, which binds to actin, hydrolyzes ATP and generates movement toward the barbed end of the actin filament. The segment of Myo X tail consists of a coiled coil, three PEST motifs, three pleckstrin homology (PH) domains, a myosin tail homology 4 (MyTH4) domain and a band 4.1/ezrin/radixin/moesin (FERM) domain.

Myo X FL is critical for the initiation and extension of filopodia, and the transport of the cargo molecules by intra-filopodial motility (Berg and Cheney, 2002; Tokuo et al., 2007, 2004). Myo X transports Mena/VASP to the tip of filopodia, and the Mena/VASP promotes

the elongation of actin filaments by interacting with the plus ends, shielding from capping proteins. The FERM domain of Myo X interacts with integrins and relocalizes integrins to the tip of filopodia (Zhang et al., 2004). This relocalization of integrins might serve to form adhesive structures and promote filopodial extension. Filopodia are motile structures linking to the enhancement of directed cell migration (Mattila and Lappalainen, 2008). In addition to actin-based motility, this unconventional myosin has a role in signal transduction. A unique feature of Myo X is the presence of three PH domains in its tail (Sellers, 2000). The second PH domain in Myo X binds phosphatidylinositol-3, 4, 5-trisphosphate [PI(3,4,5)P₃] (Tacon and Peckham, 2004). Because PI(3,4,5)P₃ is a product of the PI3-kinase, Myo X is likely to function downstream of this enzyme, an important signaling molecule in cancer and cell motility (Cantley, 2002; Comer and Parent, 2002; Ridley, 2001). A MyTH4 domain of Myo X which binds to microtubule is located next to the PH domains, and the tail of Myo X contains a C-terminal FERM domain. In addition to binding with α -integrin, Myo X also interacts with the two netrin receptors, deleted in colorectal cancer (DCC) and neogenin, an interaction dependent on the FERM domain. Myo X redistributes DCC to the cell periphery or the tips of neurites and functions in neurite outgrowth and axonal path-finding (Zhu et al., 2007). Pi et al. (2007) have reported a novel role for Myo X in endothelial cell migration, which is required to guide endothelial migration toward BMP6 gradients via the regulation of filopodial formation and amplification of BMP6 signals.

Brain synthesizes a shorter form of Myo X which lacks the myosin head domain but retains all other domains (~165 kDa). There are 3 human proteins, MAX-1, PLEKHH2 and FLJ21019 (Sousa et al., 2006) with similar structures of the motor-less Myo X, and MAX-1 is required for correct guidance of motor neurons through the netrin signaling pathway (Huang et al., 2002), implying that the headless form carries a function that is independent from the motor domain (Sousa and Cheney, 2005). However, the precise function of this unusual headless isoform of Myo X in the brain is not yet known. As the headless Myo X includes PH, MyTH4 and FERM domains, it may suppress or supplement functions of Myo X FL. This suggestion is supported by the observation that the overexpression of headless Myo X leads to defects of neurite outgrowth in primary neuron cultures (Zhu et al., 2007) and overexpression of the tail sequence from Myo X inhibits phagocytosis in a macrophage cell line (Cox et al., 2002). On the other hand, oocytes injected with Myo X tail show abnormal nuclear anchoring (Weber et al., 2004). We examined whether the headless Myo X plays a role in regulating migration of the immortalized gonadotropin-releasing hormone (GnRH) neuron *in vitro*.

The immortalized GnRH cells are widely used in studying cellular mechanisms underlying neuronal migration *in vitro* (Nielsen-Preiss et al., 2007; Cariboni et al., 2005, 2004; Allen et al., 2002; Giacobini et al., 2002; Ronnekleiv and Resko, 1990). GnRH cells are obtained from a mouse tumor in the olfactory bulb; they retain a high migratory activity (Radovick et al., 1991; Zhen et al., 1997a). We found that overexpression of the headless Myo X *in vitro* reduces the adhesion and migratory abilities of GnRH cells *in vitro*, and suggests that the headless Myo X plays a role in the neuronal migratory process.

2. Materials and methods

2.1. Antibodies and expression vectors

Antibodies were purchased as follows: anti-vinculin antibody from Sigma (St Louis, MO, USA); Rabbit polyclonal antibody anti-Myo X was generated as described (Zhu et al., 2007), as also the expression vector, pEGFP-headless Myo X. pEGFP-headless Myo X contains Myo X amino acids 727–2058.

2.2. Immunofluorescence staining procedures

NLT-type GnRH cells were cultured with high-glucose Dulbecco's minimum essential medium (DMEM) containing 10% newborn calf serum (NCS) (Gibco; New York, USA) at 37 °C in an air atmosphere with 5% CO₂. The cells were transfected using Lipofectamine 2000 (Invitrogen; Carlsbad, CA). For immunofluorescent assays, the transfected or non-transfected NLT cells were plated onto coverslips coated with 0.1 mg/ml collagen. After growth in an incubator for 5 h at 37°C in 5% CO₂, the cells were fixed with 4% paraformaldehyde for 20 min at room temperature, permeabilized with 0.1% Triton X-100 for 15 min and sequentially blocked with 2% bovine serum albumin (BSA) for 30 min in 0.01 M phosphate-buffered saline (PBS; pH 7.4). To detect focal adhesion with anti-vinculin antibody, the cells were only grown for 2 h. Coverslips were incubated for 1 h with the primary antibodies and washed three times for 15 min with PBS. They were incubated with appropriate fluorochrome-conjugated secondary antibodies for 1 h at room temperature and washed 3 times for 15 min with PBS. Images were taken on an Olympus FV1000 Viewer confocal microscope (Tokyo, Japan).

2.3. Selection of stably transfected cell lines

NLT cells were transfected using Lipofectamine 2000. The transfected cells were selected with G418 (Gibco; New York, USA, 500 µg/ml) for ~2 months until the remaining cells on the plate were all expressing the GFP-fused protein. The stably transfected cell lines were preserved for experiments. To investigate the efficiency of transfection, the stably transfected cells expressing GFP were fixed and permeabilized as described above. The nucleus was stained with Hoechst 33342 and localization of GFP was examined by fluorescence microscopy.

2.4. Immunoblotting

Cells were washed once in PBS at 4 °C and lysed in 0.2 ml RIPA buffer. The cell debris was removed by centrifugation at 12,000 *g* for 15 min at 4 °C. The protein concentration of the supernatant was determined by Coomassie brilliant blue staining. Proteins (100–150 µg) were separated on 8% SDS-PAGE gels and transferred to nitrocellulose membranes. The latter were blocked in 5% non-fat milk-PBST (0.05% Tween-20 in PBS) for 2 h at room temperature or overnight at 4 °C. Membranes were incubated for 2 h with primary antibody and washed in PBST for 45 min. The membranes were incubated with horseradish peroxidase-linked secondary antibody for 1 h, washed in PBST for 45 min, and incubated in enhanced chemiluminescence immunodetection reagents (Pierce, Rockford, IL, USA) according to the manufacturer's instructions.

2.5. Cell adhesion assay

For these assays, 24-well plates (Corning, New York, USA) were used. The wells were coated with 0.1 mg/ml collagen overnight at 4 °C and blocked with 1% BSA in PBS for 1 h. Cultured cells, at 70–80% confluency, were dissociated using trypsinization, and the cell suspension was adjusted to 3×10^5 cells/ml in DMEM containing 0.2% BSA. Cells were applied to microplate wells at 1.5×10^5 cells per well and incubated for 2 h at 37 °C. Adhesion plates with bound cells were gently washed with pre-warmed PBS to remove unattached cells. The remaining cells were fixed by 4% paraformaldehyde for 20 min at room temperature and stained with 0.2% crystal violet in 10% ethanol for 20 min (Mokhtari et al., 2008). The dye in the cells was solubilized with 10% acetic acid, and absorbance measured at 600 nm. Values from 5 independent wells for each cell line were quantified by ANOVA.

2.6. Cell aggregates and collagen gel assay

Rat tail collagen solution was prepared as described by McAteer and Cavanagh (1982). Cell aggregates were prepared by the 'hanging drop' technique (Maggi et al., 2000). The collagen solution (30 μ l) was pipetted onto the bottom of a well in a 24-well culture dish, and left to gel at room temperature. Cell aggregates were transferred onto the gel cushion and then overlaid with additional 30 μ l of collagen. After the overlaid collagen had gelled, the aggregates were covered with 1% NCS containing DMEM and cultured at 37°C in 5% CO₂ in air. The aggregates were observed daily under a light microscope. At the end of the incubation period, aggregates were fixed in 4% paraformaldehyde and phase-contrast microscope images were taken on Nikon Eclipse TE2000-U (Tokyo, Japan). The distance from the lead edge of the cell to the border of the aggregate was measured by Image J 1.41. The mean migrated distance for the cells from the same aggregate was calculated. The data obtained from 6 to 8 independent aggregates for each stably transfected cell line were compared by ANOVA analysis.

2.7. Boyden's chamber migration assays

NLT and the stably transfected cells, growing in complete media until subconfluence, were rinsed once in PBS and removed from cell culture plates by trypsin digestion. The cell suspension was centrifuged at 1000 *g* for 3 min and resuspended in DMEM medium without NCS. A total of 30,000 cells were plated into the upper chamber of Transwell. Each pair of wells was separated by a polycarbonate porous membrane (8 μ m pores) precoated with collagen (0.1 mg/ml). For chemotaxis experiments, DMEM with 1% NCS was added to the lower chamber and allowed migration for 4 h at 37 °C in 5% humidified CO₂ in air. For determination of basal migration rates, DMEM without NCS was included in the lower Transwell chamber. Following 4 h migration, the cells were fixed and stained by 0.1% crystal violet. The number of migrating cells was determined by counting cells in 10 different random fields on each membrane. The number of migrated cells, obtained from 4 independent wells for each cell line, was statistically compared by ANOVA assay.

3. Results

3.1. Expression of endogenous Myo X and an establishment of stably transfected NLT cells

Expression of endogenous Myo X in NLT cells was investigated by immunofluorescence analysis with the anti-Myo X antibody. Myo X was distributed throughout the cytosol of NLT cells and conspicuously concentrated at the tip of the filopodia (Fig. 1a). Two bands of 240 and 165 kDa were identified in Western blots with the anti-Myo X antibody, probably corresponding to Myo X FL and the headless Myo X (Fig. 1b). Stably transfected NLT cell lines overexpressing GFP-headless Myo X fusion proteins (NLT-HL) and expressing GFP (NLT-GFP) were obtained separately for determination of cell motility characteristics. Western blots of protein extracts from the two cell lines with anti-Myo X antibody revealed the presence of GFP-headless Myo X fusion proteins with an expected molecular weight of 195 kDa in NLT-HL cells (Fig. 1b). The GFP-headless Myo X was distributed in the cytosol but not in the filopodia revealed by vinculin immunolabeling (lower panel in Fig. 2a). Thus, headless Myo X is expressed in the cytosol of GnRH cells.

3.2. Overexpression of the headless Myo X reduced the cell adhesion

To see whether headless Myo X plays a role in NLT cell adhesion, focal adhesion plaques were identified with anti-vinculin antibody. Immunofluorescence assay demonstrated that NLT-HL cells contained fewer and smaller focal adhesions along the cell edges than NLT or NLT-GFP cells (Fig. 2a). The adhesion properties of NLT-HL were evaluated by the 'stick and wash' assay (Mokhtari et al., 2008). The cells were suspended and allowed to attach for

2 h before unattached cells were washed off. Many NLT cells adhered to the plate and most of them spread very well (left panel in Fig. 2b). Similarly, many NLT-GFP cells also attached to the bottom of the plate (middle panel in Fig. 2b). In contrast, fewer NLT-HL cells were observed in the bottom of the plate and, more importantly, the majority of the attached cells failed to spread with round cell shape (right panel in Fig. 2b). The attached cells were stained with crystal violet, and the cells were extracted by 10% acetic acid. The statistic data showed that the overexpression of headless Myo X resulted in a significant decrease ($P < 0.01$) in cell adhesion of NLT cells (Fig. 2c). Therefore, we concluded that the adhesive capacity of GnRH cells was reduced by the overexpression of headless Myo X.

3.3. Migration ability is inhibited by overexpression of headless Myo X

Because the overexpression of headless Myo X reduced the adhering capability of GnRH cell, we asked whether the migration ability of these cells is also affected. The collagen gel assay is a widely used assay in analyzing cell migration in 3-D matrix (Cariboni et al., 2005, 2004; Giacobini et al., 2002). Both the morphology and migrated distance of migrating cells can reliably be evaluated. The cell aggregates embedded in the collagen gel were covered by DMEM containing 1% NCS, which acts as stimulus for migration of GnRH cells. GnRH cells are able to migrate from cell aggregates into a matrix of collagen gel after the exposure to NCS. Numerous NLT and NLT-GFP cells migrated radially in chains out of the aggregate into the collagen matrix after 48 h incubation (left and middle panels in Fig. 3a), whereas fewer NLT-HL cells migrated out of the aggregate (right panel in Fig. 3a). The distance from the lead edge of the cells to the border of the aggregate was measured, and the average migrated distance was calculated. The mean migratory distances for each cell line were as follows: NLT cells ($n = 8$), $230.7 \pm 9.9 \mu\text{m}$; NLT-GFP cells ($n = 6$), $213.2 \pm 9.7 \mu\text{m}$; NLT-HL cells ($n = 8$), $162.7 \pm 25.1 \mu\text{m}$. Migration distance of NLT-HL cells was greatly reduced relative to NLT and NLT-GFP cells ($P < 0.01$). Therefore, overexpression of the headless Myo X impairs the migration of GnRH cells *in vitro*.

3.4. Chemotaxis of GnRH cells is also reduced by overexpression of GFP-headless Myo X

Subsequently, the Boyden's chamber assay was performed to investigate the role of the headless Myo X in the chemotaxis of the GnRH cells. NLT, NLT-GFP and NLT-HL cells were separately plated into the upper chamber of Transwells and allowed to migrate in the presence of 1% NCS into the lower chamber. Four hours later, the numbers of NLT, NLT-GFP and NLT-HL cells that had passed through the pores and adhered to the underside of the membrane were counted. Fewer NLT-HL cells were observed in the lower chamber (NLT cells, 221.4 ± 11.2 ; NLT-GFP cells, 221.3 ± 21.6 ; NLT-HL cells, 96.1 ± 17.9 . $n = 4$ for each; $P < 0.01$, NLT-HL vs NLT; $P < 0.01$, NLT-HL vs NLT-GFP). As to the basal migration test, all of the three cell lines showed nearly no migration in the absence of stimulus. Thus, the overexpression of headless Myo X also impairs chemotactic factors-directed migration of GnRH cells (Fig. 4).

4. Discussion

With the aid of nucleotide sequence analysis, northern blots and immunoblots, Sousa and coworkers discovered the headless form of Myo X synthesized in the brain from an alternative transcription start site (Sousa et al., 2006). Recent progress has been made in identifying domain functions of the Myo X FL for the initiation, development and transport activity of filopodia, and its critical roles in directed endothelial cell migration and axon growth have been identified (Pi et al., 2007; Zhu et al., 2007). We have investigated the role of its headless form of Myo X in the adherence and migratory ability of the immortalized GnRH neurons. The headless Myo X was expressed in NLT-type GnRH cells, and overexpression of headless Myo X impaired the adhesive and migration capacities *in vitro*.

This suggests that the headless Myo X may be implicated in the GnRH neuron migration during the brain development.

Headless Myo X with ~165 kDa is expressed in the brain, but a variety of non-neuronal cell lines including HeLa, HEK 293, COS-7, A-431, Swiss 3T3, HL-60, RAW 264.7 and LLC-PK1 cells do not express the headless Myo X, although they express Myo X FL (Sousa et al., 2006). To determine possible roles of headless Myo X in the brain, we used a neuron-derived cell line, the NLT-type GnRH cells, because they retain many morphological features of migrating neurons and expresses neuron-specific genes (Zhen et al., 1997b). Furthermore, NLT cells provide an *in vitro* model with which to explore the extracellular factors that are involved in the regulation of GnRH neuron migration as well as other cell biological events (Nielsen-Preiss et al., 2007; Allen et al., 2002; Ronnekleiv and Resko, 1990). Because the natural motor-less Myo X has the same neck and tail as the Myo X FL form, it is difficult to analyze its function by micRNA or functional antibody blocking. Therefore, we used over-expression strategy to examine the role of the headless Myo X in NLT-type GnRH cell migration *in vitro*.

Integrins are directly engaged in focal adhesion assembly, cell adhesion and the reshaping of dynamic cellular structures. Myo X interacts with integrin through the FERM domain (Zhang et al., 2004). Headless X was expressed in NLT cells and overexpression of headless Myo X in NLT cells impaired their adhesion and spreading activities. The headless Myo X contains the whole tail domain, overexpression of the headless Myo X might interfere the binding activity between integrins and Myo X FL or disturb the interaction of integrins with other FERM domain-containing proteins, such as talin that is also required for cell migration (Zhang et al., 2004). The assembly and disassembly of integrins in response to extracellular cues are essential for cell migration (Ridley et al., 2003), and thus it is likely that overexpression of headless Myo X impairs the binding activity between Myo X and integrins, and leads to reduced capabilities of adhesion and spreading of GnRH cells, which in turn contributed to impaired migration of GnRH cells (see below).

Collagen gel assay is widely used in the characterization of GnRH cell migration *in vitro*. Many factors such as HGF/SF, reelin and KAL1 play a role in guiding GnRH neuron migration (Cariboni et al., 2005, 2004; Giacobini et al., 2002). Under the same experimental conditions, the NLT-HL cells had a shorter distance relative to NLT-GFP and NLT cells, indicating that the headless Myo X suppressed cell motility in 3-D collagen gel. The Boyden's chamber assay is a procedure to evaluate cell chemotaxis. Fewer NLT-HL cells traversed the pores to the target side of the membrane than NLT-GFP or NLT cells upon stimulation with NCS, showing that the overexpression of headless Myo X inhibits the chemotaxis of GnRH cells. Thus, both spontaneous and chemotactic molecule-directed migration capabilities are reduced by the over-expression of headless Myo X.

GnRH neurons migrate in the nasal compartment intermittently in association with vomeronasal nerve fibers and increase their frequency of movement upon entering the brain, and the migration is terminated when they reach their final destinations (Tobet and Schwarting, 2006; Bless et al., 2005). The migratory route of GnRH neurons proceeds in close apposition with blood vessels, suggesting that serum factors might play distinct roles in guiding the movement of these neurons (Maggi et al., 2000). Schwarting et al. (2001) showed that DCC, the receptor of guidance factor netrin-1, regulates the trajectories of vomeronasal axons that guide the migration of GnRH neurons. Loss of DCC function results in the migration of many GnRH neurons to inappropriate destinations (Deiner and Sretavan, 1999). However, the mechanism of GnRH migration is still not well known. Myo X is believed to interact with the DCC (Zhu et al., 2007). Expression of Myo X redistributes endogenous DCC to the filopodia, and Myo X mediated filopodium formation and extension

could be further enhanced by coexpression of DCC in GnRH neurons (Zhu et al., 2007). The question raised is whether interaction between Myo X and DCC is involved in GnRH migration. Because headless Myo X decreases adhesion and migratory ability of GnRH cells *in vitro*, we speculate that it might regulate the moving state of GnRH neurons during their migratory process. Headless Myo X may promote the neurons to dissociate from the fibers by reducing the neuron adhesive ability when they come close to their target regions. Certainly, further investigations are needed to elucidate the details of these regulatory events.

Headless Myo X has the capacity to inhibit neurite outgrowth and formation of axon projections *in vivo* (Zhu et al., 2007). Migration of GnRH neurons *in vivo* is largely dependent on their interactions with vomeronasal nerve axons (Marin and Rubenstein, 2003). To distinguish the function of headless Myo X on GnRH neurons motility from their role in nerve axon projections, *in vitro* migration assays were done. Migration of GnRH cells were reduced by the overexpression of headless Myo X. Myo X FL and headless Myo X bind to several molecules that are important in neuronal development, including PI(3,4,5)P3 (Isakoff et al., 1998), microtubules (Weber et al., 2004), α -integrins (Zhang et al., 2004) and DCC (Zhu et al., 2007). Evidence from nervous and non-nervous systems suggests that headless Myo X can modify the function of Myo X FL (Zhu et al., 2007; Cox et al., 2002; Weber et al., 2004). Thus, it is likely that headless Myo X serve as a negative factor in regulating Myo X-involved GnRH neuron migration from the nasal cavity into the brain during the development. Determining the nature of this relationship of headless Myo X with Myo X FL in neuron migration is required.

In conclusion, headless Myo X suppresses adherence of GnRH cells to the matrix and decreases their migratory activity *in vitro*. These observations support a significant role of Myo X in the migration of GnRH neurons from the periphery into the brain during nervous development.

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Nomenclature

DCC	the deleted in colorectal cancer
DMEM	Dulbecco's minimum essential medium
FERM	band 4.1/ezrin/radixin/moesin
GnRH	gonadotropin-releasing hormone
MyTH4	myosin tail homology 4
Myo X	myosin X
Myo X FL	the full-length myosin X
GFP	the green fluorescence protein
NCS	newborn calf serum
NLT-GFP	the stably transfected NLT cells expressing GFP
NLT-HL	the stably transfected NLT cells expressing GFP-headless Myo X

PBS	phosphate-buffered saline
PH	pleckstrin homology
PI(3,4,5)P3	phosphatidylinositol-3,4,5-trisphosphate

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Fig. 1.

Expressions of endogenous Myo X proteins in NLT cells and establishments of stably transfected cell lines. a. Immunocytofluorescence analysis of endogenous Myo X distribution in NLT cells. Endogenous Myo X is distributed throughout the cytosol and concentrated at the tips of the filopodia. White arrow indicates the filopodia. Myo X labeled with 488 green probe (Molecular Probes, Invitrogen), bar = 25 μm.; b. Western blot analysis with anti-Myo X antibody reveals that Myo X is expressed and GFP-headless Myo X overexpressed in NLT cells. Myo X FL and the headless Myo X are expressed in the 3 cell lines, whereas GFP-headless Myo X is expressed in stably transfected cell line NLT-HL only; c. Fluorescence images of stably transfected cell line NLT-GFP and NLT-HL. Nuclei are stained with Hoechst 33342 (Blue), bar = 50 μm.

Fig. 2.

Cell adhesion assay. a. Immunocytochemical localization of adhesion complex in spread untransfected NLT cells (upper panel), NLT-GFP cells (middle panel) and NLT-HL cells (bottom panel), and focal adhesion plaques were stained with anti-vinculin antibody (red). NLT-HL cells had less adhesion plaques than wild NLT cells and NLT-GFP cells, bar = 25 μ m; b. The bright-field micrographs of the attached NLT cells, NLT-GFP cells and NLT-HL cells, bar = 100 μ m; the NLT-HL cells did not spread as well as the NLT cells and the NLT-GFP cells. The attached cells were stained with crystal violet and adhesion was quantified by measuring absorbance at 600 nm; c. Results are shown with the mean value of 5 independent measurements $\pm$ s.d. Statistical analysis indicates $**P < 0.01$.

Fig. 3.

The migration of NLT cell into a matrix of collagen gel was suppressed by overexpressing the headless Myo X. Bar = 200 μm . a. Photographs of cells NLT, NLT-GFP and NLT-HL migrating into the collagen gel from the aggregates; b. The average distance migrated per cell from every aggregate was analyzed (NLT, $n = 8$; NLT-GFP, $n = 6$; NLT-HL, $n = 8$). The NLT-HL cells migrated shorter distance than the wild NLT cells and the NLT-GFP cells. Results are the average of 6–8 independent measurements $\pm$ s.d. Statistical analysis by ANOVA indicates $**P < 0.01$.

Fig. 4.

Cell chemotaxis was inhibited by overexpressing the headless Myo X. **a**. Photomicrographs of migrated cells cultured for 4 h in the presence 1% NCS by Transwell assay. The cells migrating to the bottom side of Transwell membranes were fixed and stained with 0.1% crystal violet. Bar = 200 μ m. The average number of migrated cells per square millimeter was calculated. The number of migrated NLT-HL cells was fewer than NLT cells and NLT-GFP cells; **b**. Mean $\pm$ s.d. of migrated cells in the presence 1% NCS. (NLT, $n = 4$; NLT-GFP, $n = 4$; NLT-DN, $n = 4$). Statistical analysis by ANOVA indicates $**P < 0.01$; **c**. Mean $\pm$ s.d. of cells migrated in the absence of NCS. (NLT, $n = 5$), (NLT-GFP, $n = 5$) and (NLT-DN, $n = 5$).