Expression of ErbB Receptors and their Cognate Ligands in Gastric and Colon Cancer Cell Lines

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Abstract. *Background: ErbB receptors and their cognate ligands are implicated in cancer progression. Their expression in gastrointestinal cancer, however, has not been systemically studied. Materials and Methods: The expression of four ErbB receptors and a panel of ErbB ligands were determined by reverse transcription-PCR in two gastric (TMK1, MKN-45) and two colon (SW1116, HT-29) cancer cell lines. Cell proliferation was measured by MTT assay while gene knockdown was achieved by RNA interference. Results: ErbB1, ErbB2 and ErbB3 receptors and five known or putative ErbB ligands, namely, epiregulin, epidermal growth factor (EGF), heparin-binding EGF, transforming growth factor α (TGFα) and neuroglycan-C were expressed in all four cell lines. Knockdown of neuroglycan-C, however, did not affect cell proliferation. Conclusion: This study profiles the expression of ErbB receptors and their cognate ligands in gastric and colon cancer cells. These findings might lay the basis for the development of ErbB pathway-directed therapeutics for gastrointestinal cancer.*

Mitogenic signals transduced by receptor tyrosine kinases (RTKs) play an important role in the pathogenesis of human cancer (1). Among all RTKs, the ErbB receptor family has up to now received the greatest attention owing to the finding that their expression is up-regulated in many epithelial tumors (2, 3). A large number of pre-clinical studies also confirm that ErbB receptor-mediated signaling directly contributes to cell proliferation (4), resistance to apoptosis (5), angiogenesis (6), and invasion and metastasis (7), all of which are related to tumor progression. The ErbB receptor family encompasses four members, namely, ErbB1/epidermal growth factor (EGF) receptor, ErbB2/neu/HER2, ErbB3/HER3 and ErbB4/HER4 (8). Although all four ErbB receptors have different ligand-binding properties, they are believed to share an overlapping downstream signaling network. Upon binding of ligands, the ErbB receptors form homodimers or heterodimers with other ErbB family members. The dimerized receptors are then internalized and autophosphorylated on their tyrosine residues which trigger diverse downstream signaling pathways including the Ras/mitogen-activated protein kinases (MAPKs) (9), the Janus kinase-signal transducers and activators of transcription (JAK-STAT) (10), the phospholipase Cγ (11), and the phosphatidylinositol 3-kinase/Akt pathways (12). It is also worthwhile to note that ErbB2 has a functionless ligand-binding domain while ErbB3 exhibits a defective tyrosine kinase (8).

One of the strategies employed by cancer cells to maintain constitutively active RTKs is autocrine secretion of RTK ligands (2). The ligands for ErbB receptors are numerous and diversified and include EGF, transforming growth factor α (TGFα), heparin-binding EGF (HB-EGF), amphiregulin, betacellulin, epiregulin, epigen, neuregulins and neuroglycan-C (13). Recent sequence analysis studies further reveal additional putative ErbB ligands whose amino acid sequences share homology with the evolutionarily conserved EGF motifs. These putative ErbB ligands include meprin, interphotoreceptor matrix proteoglycan-2, and mucin-3, -4, -12 and -17 (13). Most of the known or putative ErbB ligands are expressed as transmembrane precursors in which protease-mediated cleavage is required to release the mature ligands (14).

Aberrant expression levels of ErbB receptors and their cognate ligands have been recognized as one of the causes of cancer progression (1, 2). Moreover, it has been proposed that the efficacy of ErbB receptor-targeted anticancer therapeutics is influenced by the availability of ErbB ligands (15). Failure of some therapeutic monoclonal antibodies that target a particular ErbB receptor has also been attributed to

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the co-expression of other ErbB receptors (16). For instance, it has been suggested that ErbB1/ErbB2 heterodimer formation may contribute to the development of resistance to trastuzumab, a monoclonal antibody against ErbB2, in a subset of breast cancer patients (16). A comprehensive analysis to profile the expression of ErbB receptors and ligands is therefore important to the elucidation of the pathogenic mechanisms and the application of ErbB signaling-directed therapeutics. Among all types of cancer, adenocarcinomas of the gastrointestinal tract account for a large proportion of cancer-related mortality (17). Nevertheless, there are only sporadic reports in the literature to account for the expression of ErbB ligands and their receptors. Moreover, cetuximab, a chimeric anti-ErbB1 monoclonal antibody, has been in clinical use for the treatment of metastatic colorectal cancer (18). The expression of ErbB receptors and ligands in gastrointestinal cancer, however, remains poorly understood. The aim of this study was therefore to investigate the expression of ErbB receptors and their cognate ligands in gastric and colon cancer cells. This may lay the basis for the development of ErbB pathway-directed therapeutics for gastrointestinal cancers.

Materials and Methods

Cell culture. The gastric adenocarcinoma cell line TMK1 was obtained from Dr. Eiichi Tahara (University of Hiroshima, Hiroshima, Japan). The human gastric adenocarcinoma cell line MKN-45 and colon adenocarcinoma cell lines, SW1116 and HT-29, were purchased from American Type Culture Collection (Manassas, VA, USA). All four cell lines were maintained in RPMI-1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen), 100 U ml^{-1} penicillin G and 100 μg ml^{-1} streptomycin, and maintained at 37˚C, 95% humidity and 5% carbon dioxide.

Conventional and quantitative reverse transcription-polymerase chain reaction. The total cellular RNA was isolated using Trizol reagent (Invitrogen). The RNA concentration was measured by SmartSpec Plus spectrophotometer (Bio-Rad, Hercules, CA, USA) at 260 nm. The same amount of total RNA (5 μg) was used to generate the first strand of cDNA by reverse transcription (Invitrogen) in accordance with the manufacturer's instructions. Specific primers (Table I) were designed to screen the expression of ErbB receptors and their ligands. Forward and reverse primers were designed to bind to different exons of the same gene to avoid false-positive signals from genomic DNA amplification. The PCR conditions were as follows: the template cDNA was first denatured at 94˚C for 5 min. During 35 cycles of amplification, the denaturation step was at 94˚C for 1 min, the annealing step at 55˚C for 1 min (see Table I) and the extension step at 72˚C for 1 min. The final extension step was at 72˚C for 7 min. The PCR products were electrophoresed on a 1.0% agarose gel containing 1x GelRed reagent (Biotium, USA) and visualized under UV-transillumination using ChemiDoc XRS system (Bio-Rad). For the quantitation of neuroglycan-C expression, real-time PCR was performed with specific primers (Table I). Conditions for quantitative PCR were 94˚C for 5 min, 45 cycles of 94˚C for 30 s, 55˚C for 30 s and 72˚C for 30 s. Quantitative PCR was carried out using CyberGreener real-time PCR supermix (Invitrogen) and iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad) as recommended by the manufacturer. The results were analyzed using the comparative threshold cycle (C_T) method with β-actin as an internal control. The specificity of PCR product was confirmed by melting-curve analysis and DNA gel electrophoresis.

RNA interference. The expression of neuroglycan-C was lowered using pre-designed target-specific small interference RNA (siRNA) molecules purchased from Qiagen (Valencia, CA, USA). Ten picomoles per well of gene-specific or control siRNA was transfected into TMK1 cells seeded on a 96-well microplate at 40-60% confluence using Lipofectamine™ 2000 reagent (Invitrogen) according to the manufacturer's instructions. Messenger RNA was extracted 48 h post-transfection from TMK1 cells for RT-PCR to determine the RNA interference efficacy. The transfection efficiency was monitored using florescent RNA duplex (Invitrogen) according to the manufacturer's instructions.

MTT cell proliferation assay. Transfected TMK1 cells (3.0×10^3 cells/well) in 96-well culture plates were incubated up to 4 days. At different time points (48 h- and 72 h-post-transfection), 10 μl of MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] solution (5 mM; Sigma) was added to each well and the plates were incubated for another 2 h. Dimethyl sulfoxide was then added to solubize MTT tetrazolium crystal. Finally, the optical density was determined at 570 nm using a Benchmark Plus microplate reader (Bio-Rad).

Statistical analysis. Results were expressed as the mean±SEM. Statistical analysis was performed with student's *t*-test. *P*-values less than 0.05 were considered statistically significant.

Results

Gastric and colon cancer cell lines expressed ErbB1, ErbB2 and ErbB3 receptors. As ErbB receptors have been implicated in the pathogenesis of gastric and colon cancer (19, 20), the mRNA expression of four members of the ErbB family of receptors was screened in two gastric (TMK1, MKN45) and two colon (HT-29, SW1116) cancer cell lines using RT-PCR. It was found that mRNA expression of ErbB1, ErbB2 and ErbB3 receptors was detected in TMK1, MKN45, HT-29 and SW1116 (Figure 1). Notably, ErbB3 receptor was strongly expressed in all four cell lines. In contrast, the expression of ErbB4 receptor was only detectable in SW1116.

Gastric and colon cancer cells expressed epiregulin, EGF, HB-EGF, TGFα and neuroglycan-C. As ErbB1, ErbB2 and ErbB3, but not ErbB4 receptors appeared to be commonly expressed in gastric and colon cancer cells, the mRNA expression of their cognate ligands were determined. Results showed that amphiregulin, epiregulin, EGF, HB-EGF, TGFα

Table I. *Oligonucleotide sequence of primers used for RT-PCR.*

ErbB Receptors

Gene name		Primer sequence	Binding exon no.	Product size (bp)
ErbB 1	Forward	CTACAACCCCACCACGTACC	7	411
	Reverse	CAGCTCAAACCTGTGATTTCC	12	
ErbB 2	Forward	CCCTCTGACGTCCATCATCT	20	698
	Reverse	CCCCATCTGCATGGTACTCT	24	
ErbB 3	Forward	GATTTTGGTGTGGCTGACCT	21	573
	Reverse	ACGTGGCCGATTAAGTGTTC	25	
ErbB 4	Forward	TACCGAGATGGAGGTTTTGC	27	447
	Reverse	GTTGGCAAAGGTGTTGAGGT	28	

ErbB Ligands

Gene name		Primer sequence	Binding exon no.	Product size	Receptor binding
Amphiregulin	Forward	AAAAAGGGAGGCAAAAATGG	3	457	E1
(AREG)	Reverse	TGGAAAGAGGACCGACTCAT	6		
Betacellulin	Forward	TTCACTGTGTGGTGGCAGATGG	2	350	E1, E4
(BTC)	Reverse	ACAGCATGTGCAGACACCGATG	4		
Epigen	Forward	AGGAAATGGCTTTGGGAGTT	1	685	E1
(EPGN)	Reverse	CTACTTTCTTGGGGCCTGCT	4		
Epiregulin	Forward	TCCAGTGTCAGAGGGACACA	1	488	E1, E4
(EREG)	Reverse	GGTTGGTGGACGGTTAAAAA	4		
Epidermal growth	Forward	CCAGCGAGAAAGGCTTATTG	20	514	E1
factor (EGF)	Reverse	ATGGCATAGCCCAATCTGAG	24		
Heparin-binding	Forward	GGCAGATCTGGACCTTTTGA	2	549	E1, E4
EGF (HB-EGF)	Reverse	ACTGGGGACGAAGGAGTCTT	6		
Meprin 1α	Forward	GAAATCCAGAAATGGCCTGA	5	496	Un
(MEP1α)	Reverse	GGTGGGAACACTTGCATTCT	8		
Meprin 1β	Forward	TGTGTGGCATGATCCAAAGT	9	466	Un
(MEP1β)	Reverse	CACCGTGTTTCCGAAAGATT	11		
Interphoto-receptor matrix	Forward	TGGGTTACCAGGCTACAAGG	8	669	Un
proteoglycan-2 (IMP2)	Reverse	CTTTCCTCAGAAGCCACAGG	12		
Mucin 3	Forward	TACAGACATCCCGACCACAA	11	461	Un
(MUC3)	Reverse	GCCTGGGAAGTGTTGTCATT	14		
Mucin 4	Forward	CTTCACAGACAATGGCCAGA	3	490	Un
(MUC4)	Reverse	GGACATCAGTGGGCTGTTTT	6		
Mucin 12	Forward	ACCTTAGCACCAGGGTTGTG	4	603	Un
(MUC12)	Reverse	CAGCTTCCCATCCAAGACAT	10		
Mucin 17	Forward	TGTGCCAAGAACCACAACAT	3	434	Un
(MUC17)	Reverse	TCACTACTTCGGTGGCATTG	7		
Transforming growth	Forward	TCGCTCTGGGTATTGTGTTG	1	462	E1
factor α (TGFα)	Reverse	TCTGGGCTCTTCAGACCACT	6		
Neuroglycan-C	Forward	CCCCACCACATCCTTTTATG	2	680	E3
(NGC)	Reverse	GGGGAGCACTAGGATCATCA	5		
Neuregulin-1	Forward	AAGAAGGACTCGCTGCTCAC	---	515	E3, E4
(NRG1)	Reverse	CGGCACATTTCCGATCTACT	---		
Neuregulin-2	Forward	CGTTGGTAAAGGTGCTGGAC	1	586	E3, E4
(NRG2)	Reverse	ACGCAATAGGACTTGGCTGT	4		

E1: ErbB1; E2: ErbB2; E3: ErbB3; E4: ErbB4; Un: unidentified.

and neuroglycan-C were expressed in both TMK1 and MKN-45 gastric cancer cells (Figure 2A). The expression of ErbB ligands in colon cancer cells demonstrated a similar pattern except that amphiregulin expression was not detected in SW1116 cells. Moreover, a number of putative ErbB ligands, including meprin-1α/β, interphotoreceptor matrix proteoglycan-2, mucin-12 and -17, and neuregulin-2 were expressed in both HT-29 and SW1116 cells (Figure 2B).

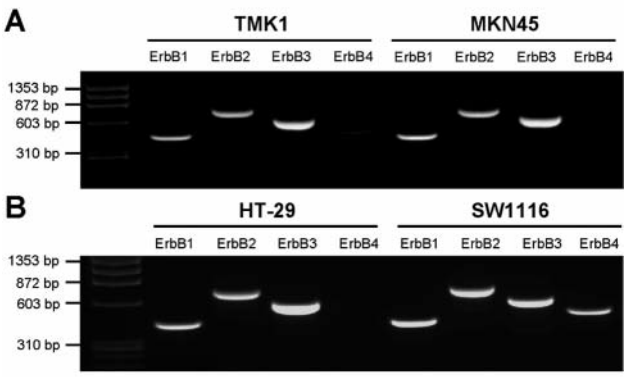

Figure 1. *Reverse transcription-PCR revealed the expression of ErbB1, ErbB2 and ErbB3 receptor mRNA in the gastric adenocarcinoma cell lines, TMK1 and MKN45, and colon adenocarcinoma cell lines, HT-29 and SW1116. The expression of ErbB4 mRNA was only detected in SW1116. The amplicon size of ErbB1, ErbB2, ErbB3 and ErbB4 was 411 bp, 698 bp, 573 bp and 447 bp, respectively.*

Knockdown of neuroglycan-C on TMK1 cell proliferation. Aberrant ErbB ligand expression is associated with tumor progression (21). In this respect, the contributory roles of epiregulin, EGF, HB-EGF and TGFα to the proliferation of gastric or colon cancer cells has been reported in many studies (22-25). The effect of endogenous neuroglycan-C on gastrointestinal cancer cell proliferation, however, has not yet been described. The RNA interference approach was therefore employed to deplete endogenous neuroglycan-C expression and the effect of neuroglycan-C knockdown on proliferation of TMK1 cells was determined. In this set of experiments, siRNA transfection efficiency was determined to be more than 85% (Figure 3A) and neuroglycan-C-siRNA significantly reduced the mRNA expression of neuroglycan-C by 80% at 48 h post-transfection (Figure 3B). Nevertheless, growth curve analysis by MTT proliferation assay suggested that knockdown of neuroglycan-C did not affect cell proliferation compared with control siRNA-transfected cells at 48 h or 72 h post-transfection (Figure 3C).

Discussion

ErbB signaling plays a crucial role in the regulation of many pivotal cellular processes, some of which are related to carcinogenesis, including proliferation, differentiation and apoptosis (4-7). It therefore comes as no surprise that up-regulation of ErbB signaling has been reported in many epithelial cancers. In this study, it is demonstrated that ErbB1, ErbB2 and ErbB3 receptors are commonly expressed in gastric and colon cancer cell lines. These findings are in line with previous studies showing that these receptors are expressed in gastric and colon cancer. Moreover, the present data indicate that the expression of ErbB4 is not detectable in gastric cancer cells, which is in line with the findings of Noguchi *et al.* (19). In colon cancer cells, ErbB4 expression is only detected in SW1116 but not HT-29. These data indicate that ErbB1, ErbB2 and ErbB3, but not ErbB4, are predominantly involved in gastrointestinal carcinogenesis.

One of the common strategies harnessed by cancer cells to maintain persistent mitogenic stimulation is co-expression of ErbB receptors and their cognate ligands (8). In this study, it is shown that some classical ErbB ligands, including epiregulin, EGF, HB-EGF and TGFα are ubiquitously expressed in gastric and colon cancer cells, suggesting autocrine stimulation of these ligands on their respective receptors may be involved in the maintenance of basal cell proliferation. To this end, it is known that epiregulin and HB-EGF bind to both ErbB1 and ErbB4 whilst EGF and TGFα only bind to ErbB1 receptor (13). Given that ErbB4 receptor is not expressed in most gastric and colon cancer cell lines, this finding may reflect the scenario that ErbB1 subtype is the key player in mediating ErbB signaling in gastrointestinal cancer. Moreover, aside from the expression of these classical ligands, the study also reveals for the first time that colon cancer but not gastric cancer cells express an array of putative ErbB ligands, including meprin-1α/β, interphotoreceptor matrix proteoglycan-2, mucin-12 and -17, and neuregulin-2. Previous studies utilizing phylogenetic analysis reveal that these proteins share sequence homology with that of EGF (13) but the molecular identities of their cognate receptor and their cellular function in relation to colon carcinogenesis, however, has not yet been determined.

Neuroglycan-C was originally identified as a transmembrane chondroitin sulfate proteoglycan specifically expressed in the brain (26). Later studies further revealed that neuroglycan-C contains an EGF-like domain which enables direct binding for ErbB3 (27). Neuroglycan-C also induces human breast cancer cell proliferation in which phosphorylation of ErbB2 and ErbB3 is observed (27). In the present study, the results demonstrate that neuroglycan-C is highly expressed in gastric and colon cancer cells. Depletion of endogenous neuroglycan-C expression, however, does not affect cell proliferation, indicating that the function of this novel ErbB3 ligand might be different from that reported in other cell types. Nevertheless, whether knockdown of neuroglycan-C could affect other cellular processes such as cell survival, invasion and metastasis, and angiogenesis, however, requires further elucidation.

Autocrine stimulation of ErbB ligands on their receptors is involved in accelerated cell proliferation (1, 2) and development of drug resistance to ErbB-directed cancer therapeutics (15, 16). Here the expression of ErbB receptors and their cognate ligands in gastric and colon cancer cells was comprehensively profiled. These findings might help to better understand how ErbB signaling is deranged in gastrointestinal cancer and hopefully expand the current list of targets for the development of ErbB pathway-directed therapeutics.

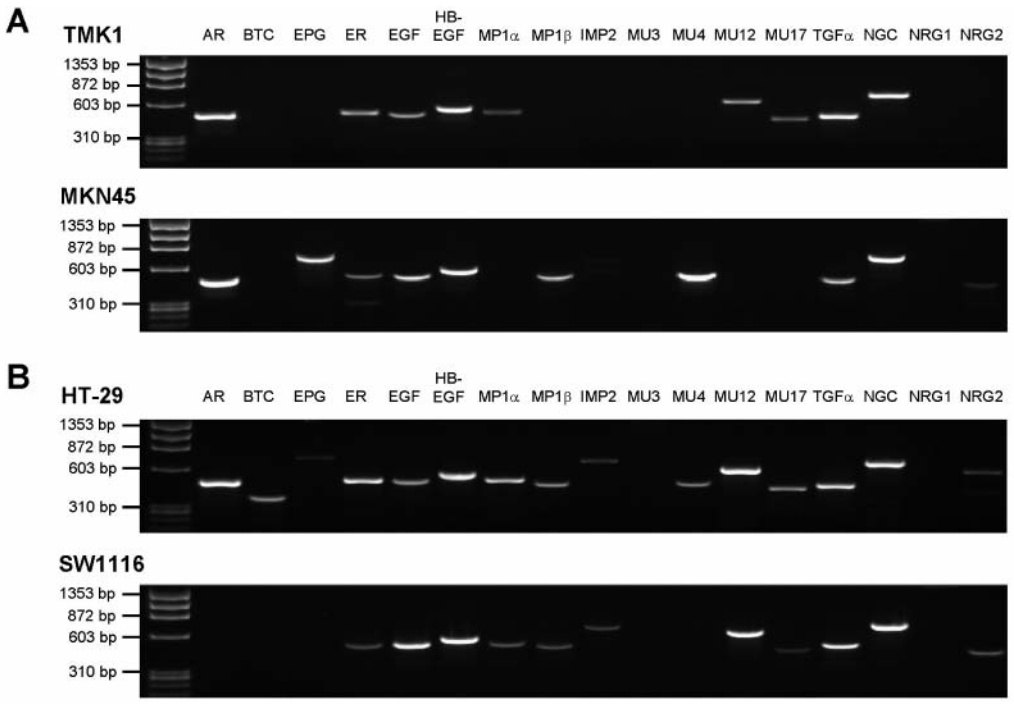

Figure 2. *The expression of a collection of known or putative ErbB ligands in gastric and colon cancer cell lines were determined by reverse transcription-PCR. Epiregulin, EGF, HB-EGF, TGFα and neuroglycan-C were expressed in all four cancer cell lines. Moreover, the expression of an array of putative ErbB ligands was also detected in colon cancer cell lines HT-29 and SW1116.*

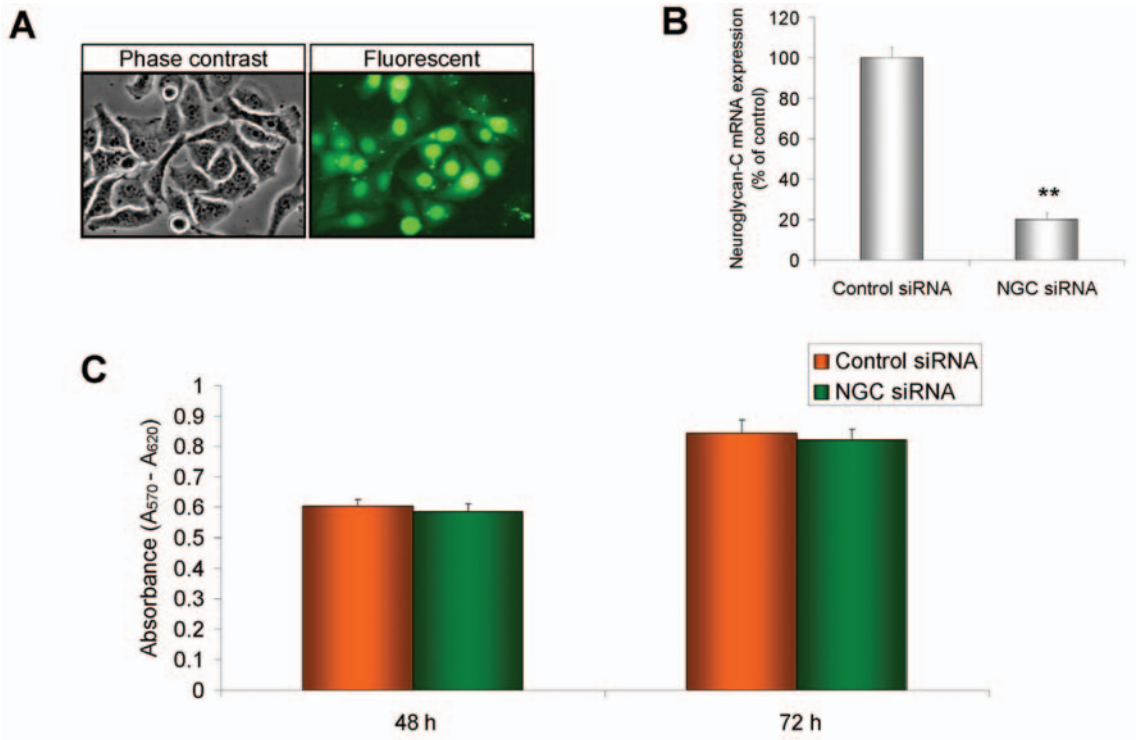

Figure 3. *The effect of siRNA-mediated depletion of endogenous neuroglycan-C on TMK1 cell proliferation was determined by MTT assay. A, Transfection efficiency was determined by transfecting TMK1 cells with fluorescent-labeled RNA duplex. B, Neuroglycan-C siRNA significantly reduced the mRNA expression in TMK1 cells, which was determined 48 h post-transfection. C, Knockdown of neuroglycan-C did not alter cell proliferation at 48 h or 72 h post-transfection. **p<0.01, Significantly different from control siRNA-transfected group.*

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