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Summary

CSPG4/NG2 is a multifunctional transmembrane protein with limited distribution in adult tissues including articular cartilage. The purpose of the current study was to investigate possible roles for CSPG4/NG2 in chondrosarcomas and to establish whether this molecule may have potential for targeted therapy. Stable knock down of CSPG4/NG2 in the JJ012 chondrosarcoma cell line by shRNA resulted in decreased cell proliferation and migration as well as a decrease in gene expression of the MMP (matrix metalloproteinase) 3 protease and ADAMTS-4 aggrecanase. Chondrosarcoma cells in which CSPG4/NG2 was knocked down were more sensitive to doxorubicin than wild type cells. The results indicate that CSPG4/NG2 has roles in regulating chondrosarcoma cell function in relation to growth, spread and resistance to chemotherapy and that anti-CSPG4/NG2 therapies may have potential in the treatment of surgically irresectable chondrosarcoma.

Keywords: CSPG4, NG2, Chondrosarcoma, Therapy

Introduction

Chondrosarcoma is the most common primary malignant bone tumour in adults over the age of 30, the incidence increasing with age. Prognosis depends largely on the histological grade. Low grade lesions are matrix rich with low cellularity. Higher grade tumours show increased cellularity, mitotic activity and cellular pleomorphism (Evans*, et al.*, 1977). Conventional chondrosarcomas account for the vast majority and approximately 90% of these are low- to intermediate-grade tumours characterised by indolent clinical behaviour and low metastatic potential (Gelderblom*, et al.*, 2008). Nevertheless the clinical management of chondrosarcoma remains a challenging problem. Disease free survival and overall survival of patients with chondrosarcoma have not shown any significant improvement over the last few decades. Whilst surgical resection remains the treatment of choice for the majority of chondrosarcomas and complete resection of low to intermediate-grade tumours is often curative, there are a significant number of chondrosarcomas, especially those arising in the pelvis or the spine that are difficult to remove with a wide surgical margin (Bergh*, et al.*, 2001, Bruns*, et al.*, 2001). In these instances local recurrence is almost inevitable and becomes a serious clinical problem.

Although they may be used in treatment of metastatic or unresectable tumours, radiotherapy and chemotherapy are on the whole ineffective in the management of chondrosarcoma. Recent advances in the understanding of the biology of chondrosarcomas have indicated potential targets for new therapies. Somatic heterozygous missense mutations in the genes encoding isocitrate dehydrogenase (IDH)1 or IDH2 are frequent genetic events in low-grade and high-grade chondrosarcomas(Amary*, et al.*, 2011) *a*nd compounds that specifically target mutant IDH1 R132H and IDH2 R140Q have been developed (Popovici-Muller*, et al.*, 2012). Therapies aimed at regulating activity of molecules and pathways involved in chondrosarcoma cell death/survival, proliferation or migration are also gaining interest (Dai*, et al.*, 2011, Jamil*, et al.*, 2010). Targeting specific molecules involved in chondrosarcoma

cell proliferation, migration and cell survival have the potential to improve outcomes in those patients with chondrosarcomas who have inoperable, recurrent and metastatic disease.

One such candidate molecule is CSPG4/NG2. CSPG4/NG2 is a transmembrane protein expressed by cells typically as both a 250kD glycoprotein and a higher molecular weight, 400-450kD proteoglycan (Stallcup, 2002). Initial reports suggested that in normal human adult tissues CSPG4/NG2 expression is restricted to a limited number of cell types including vascular pericytes, articular chondrocytes, microglia in the central nervous system and their neoplastic counterparts such as oligidendrogliomas, glioblastomas and chondrosarcomas. However it is now clear that CSPG4/NG2 is expressed in a broader range of tissues and tumours types such as childhood acute lymphoblastic leukemia, malignant melanoma, renal cell carcinomas, pancreatic cell carcinomas, triple-negative breast cancer and squamous cell carcinoma of the head and neck (Stallcup and Huang, 2008, Wang*, et al.*, 2010). Importantly expression of CSPG4/NG2 is associated with tumour progression in high grade pleomorphic soft tissue sarcomas where metastases show greater than 80 fold increases in gene expression levels when compared to primary tumours (Benassi*, et al.*, 2009, Cattaruzza*, et al.*, 2013). The aim of the current study was to investigate possible roles for CSPG4/NG2 in chondrosarcomas and to establish whether this molecule may have potential for targeted therapy.

Methods

Chondrocyte cell lines and tissues

The JJ012 (grade II chondrosarcoma) cell line was kindly gifted by Professor Joel A. Block, Rush University Medical Centre, Chicago, USA. Articular cartilage was donated by patients undergoing knee replacement surgery. Chondrosarcoma samples were obtained at the time of initial bone tumour biopsy or at definitive surgical resection. Ten chondrosarcoma samples were obtained from the archive of the Department of Pathology, Lothian University Hospital Trust. These were from 7 males and 3 females, age 21-77, and included 4 grade 2 chondrosarcomas from the pelvis, 2 grade 2 chondrosarcomas from the scapula and calcaneus, a grade 3 chondrosarcoma from the proximal femur, a grade 2 pelvic chondrosarcoma metastatic to the lung, a grade laryngeal chondrosarcoma and a distal femoral clear cell chondrosarcoma. Patient samples were obtained with informed consent and ethical approval.

CSPG4/NG2 gene knockdown in JJ012 cells was by shRNA (MISSION® shRNA Library , Sigma-Aldrich UK). In brief, five different constructs against CSPG4/NG2 were used together with GFP and non target controls. Cells were transfected by a serial 10 fold dilution of viral particles, following which they were cultured in medium containing puromycin. Resistant clones were expanded in medium containing puromycin and expression of CSPG4/NG2 assessed. Cells with maximum gene knock down (B3 cells), as assessed by RTPCR and western blotting, were used in subsequent experimental studies.

Immunohistochemistry

Sections were cut from formalin fixed, paraffin embedded blocks, dewaxed in xylene before being rehydrated in decreasing concentrations of alcohols. Antigen retrieval was achieved through incubation of samples in 10 mM sodium citrate solution at 95^{o}C for 25 minutes. Sections were then treated in 3% H_2O_2 solution for 10 minutes. Blocking and antibody incubation steps were performed

in accordance with the Envision system (Dako, UK) instructions. Sections were immunostained stained using anti-CSPG4/NG2 rabbit polyclonal antibody ab86067 (Abcam, UK) at a concentration of 1:200. Diaminobenzidine solution was freshly prepared and added for nine minutes. Slides were countered stained in Haematoxylin and then dehydrated in increasing concentrations of alcohol before coverslips were mounted.

Cell proliferation*, *adhesion and spreading assays

Cell proliferation was assessed by using both the CyQUANT® Cell Proliferation Assay kit (Invitrogen, UK) according to the manufacturer's instructions and the MTT assay (Peng, *et al.,* 2012). Cell adhesion was assessed using methylene blue staining as described previously (Midwood and Salter, 1998). In brief, 96 well plates were coated with 10µg/ml of type I, II or VI collagen, human fibronectin or bovine serum albumin (BSA) in 50µl PBS overnight at 4^{o}C. Cells were seeded at 2.0 x10^{4}/100µl in triplicate and allowed to adhere for 1hr at 37^{o}C. After fixing in 4% formaldehyde, cells were stained in 1% methylene blue for 30min. Adsorbed methylene blue was solubilized in 0.1N hydrochloric acid, mixed thoroughly, and the plate read at 630 nm on an ELISA reader (MR5000, Dynatech UK). Assessment of cell spreading was undertaken by allowing cells to adhere to 96 plate wells, coated with either collagen types I, II, VI or fibronectin for 1hr at 37^{o}C before fixing in 4% formaldehyde and imaging using a bright field microscope. Images were taken from at least three independent areas per well and the number of spreading cells as previously described(Humphries, 2000) were counted.

Wound healing and inverted cell invasion assays

A wound healing assay was used to assess cell migration in monolayer. Cells were seeded in 12 well plates at 7.5x10^{4}/well and grown until near confluent. A wound was made through the cells at the middle of the wells by scratching with a micropipette tip. The wells were then washed carefully with media. The area of wounding was imaged using bright field imaging immediately after and 10 hours after the scratch. The percentage of area closed was assessed using T scratch software. For the inverted cell invasion assay 100 µl of growth factor reduced Matrigel (BD Bioscience, UK) was pipetted

into each transwell of a 24 well plates and incubated for 30min at 37^{o}C. When the Matrigel was set, 100 µl of cell suspension (10^5 cells/ml) was pipetted onto the underside of the filter of the inverted transwells and incubated for 4 hours. After washing the transwells 3x with serum free medium, 100 µl of either: serum containing media or serum containing media + Epidermal Growth Factor (EGF) was pipetted gently into the transwell on top of the matrigel. After 5 days the matrigel was stained with Calcein 4µM (Invitrogen, UK) and imaged by confocal microscopy. Optical sections were scanned at 15µm intervals moving from the underside of the filter into the Matrigel and quantified using "Image J" software.

Molecular studies and western blotting and flow cytometry

RNA was extracted from cultured cells using an RNAse Mini kit (Qiagen, UK) following the manufacturer's instructions and quantified using a Nanodrop1000 spectrophotometer (Thermo Scientific, UK). RT-PCR was carried out using Qiagen Long Range 2 step RT- PCR kit with a (G-Storm) thermal cycler. The qPCR reaction was carried out using SYBR Green and the Applied Biosystems 7500 thermal cycler. RNA integrity testing and genomic DNA contamination steps were performed prior to RT-PCR and qPCR reactions.

RIPA buffer with protease inhibitors was used to extract protein from cells for western blotting. In some studies protein extracts were incubated with chondroitinase ABC at 1U/ml for 45 minutes at room temperature. Following western blotting, the membrane was incubated with 5% Marvel, washed with TBST and incubated with 1 µg/ml primary antibody, LHM2 (sc-53389, Santa Cruz Biotechnology USA) overnight at 4^{o}C. The membrane was washed again and incubated with secondary antibody for 1hr at RT before further washes, incubation with ECL plus (Amersham, UK) and visualisation using a VersaDoc^{TM} imaging system (BIORAD, UK). Alpha tubulin was used as a positive control for protein loading.

For flow cytometry 2x10^6cells were incubated with 50µl of FACS wash solution and mouse serum on ice for 15 minutes before incubation primary antibodies for 30 minutes at 4^{o}C. Cells were then washed

and centrifuged for 5 minutes three times. Samples were mixed with 200µl FACS wash buffer and analysed using the FACS Calibur (BD, UK). For Annexin V staining, cells were resuspended in Hanks balanced salt buffer/Annexin V and incubated on ice for 10-15minutes before analysis using a FACscan system (BD, UK).

Statistical analysis

The mean, standard deviation and standard error of the mean (SEM) were determined in each experiment. In each experiment, the samples were tested whether they are normally distributed or not. Student unpaired t-test was used to identify statistically significant differences between two independent groups. For three independent groups, one-way ANOVA was used. The difference was considered statistically significant when $p<0.05$.

Results

CSPG4/NG2 expression in the JJ012 chondrosarcoma cell line

Gene expression of CSPG4/NG2 in the JJ012 cell line was similar to that of human articular chondrocytes and the transformed chondrocyte cell line C20 A4 as assessed by Q-PCR (results not shown). Western blotting showed that CSPG4/NG2 was expressed as a single, non-chondroitinase sulphated protein of approximately 270kD in the JJ012 cell line whereas articular chondrocytes expressed a predominantly chondroitinated proteoglycan of high molecular weight (Fig 1a). Immunofluoresence of CSPG4/NG2 distribution in JJ012 cells in monolayer culture showed predominantly cell membrane expression with focal enhancement as discrete projections (Fig 1b). In contrast articular chondrocytes showed both cell membrane and cytoplasmic expression in monolayer culture (Fig 1b). All ten chondrosarcoma samples showed strong staining of the chondrosarcoma cells. The staining was most pronounced at the cell membrane but in all cases there was weaker, more diffuse cytoplasmic staining (Fig 1c). In cases where adjacent soft tissue was present only pericytic cells were seen to show positive staining

Generation of a stable CSPG4/NG2 knockdown JJ012 cell line

CSPG4/NG2 knockdown was carried out using the Mission TRC shRNA lentiviral transduction method. Five different constructs against CSPG4/NG2 (A, B, C, D and E) together with the negative (shRNA non-target) and the positive (shRNA GFP) controls were used. The gene knockout efficiency was confirmed by QPCR and western blotting. QPCR showed that D1 and B3 constructs had the highest CSPG4/NG2 gene knockdown efficiency among all colonies selected (Fig 2 upper panel). Western blotting confirmed decreased expression of CSPG4/NG2 in both B3 and D1 cells (Fig 2 lower panel). As protein expression in B3 cells appeared to be minimal this cell line was selected for use in subsequent studies.

CSPG4/NG2 knock-down is associated with decreased cell proliferation

To assess the role of CSPG4/NG2 in chondrosarcoma cell proliferation, JJ012 (NG2+ve), B3 (NG2 -ve) cells and non-target cells were grown in culture for 24 and 48 hours and cell numbers assessed by two different cell proliferation assays. With the CyQUANT® Cell Proliferation assay, B3 cell numbers were significantly lower in comparison to those of JJ012 cells following 48 hours in monolayer culture, whilst there was no difference in cell numbers between JJ012 cells and non-target (Fig 3a upper panel). Similar results were obtained with the MTT assay (not shown). To establish whether the increase in cell number following culture was a consequence of differences in cell proliferation and not cell death, annexin V expression, a marker of apoptosis, was assessed at 24 and 48 hours in culture. No significant difference in the percentage of annexin V positive cells in JJ012, B3 and non-target cell cultures was seen (Fig 3a lower panel) indicating that the difference in B3 and JJ012 cell numbers seen after 48 hour culture was most likely a consequence of differences in cell proliferation.

Roles for CSPG4/NG2 in chondrosarcoma cell adhesion, spreading, migration and invasion.

Both JJ012 (CSPG4/NG2 +ve) and B3 (CSPG4/NG2 -ve) cells adhered to collagen I, II, VI and fibronectin coated wells. There was no difference in adherence of JJ012 to the different matrix molecules whereas B3 cells appeared to show greater adherence to fibronectin in comparison to collagen ($p=0.048$) (Fig 3b. upper panel). There was a trend for adherence of B3 cells to collagen II, VI and fibronectin coated wells to be greater than that of JJ012 cells although this did not reach statistical significance. There was no difference in the spreading of either JJ012 and control cells (non-target, CSPG4/NG2 +ve) cells on the different matrices. In contrast a statistically greater increase in spreading of B3 cells on collagen I, II, VI and FN ($p<0.0001$; $p<0.008$; $p<0.0008$ $p<0.0003$ respectively) was seen (Fig 3b lower panel). In the wound healing assay, 10 hours following wounding in a monolayer culture, the closure of the wound area by B3 cells (54.6%) was significantly less ($p=0.002$) than that of both JJ012 cells (81.4%) and the non-target control cells (82.2%) (Fig 4a). In contrast, there was no difference identified in the extent of invasion of the B3 and JJ012 cells into Matrigel in the inverted cell adhesion assay in the absence or presence of EGF as a chemoattractant (Fig 4b).

The effect of CSPG4/NG2 knockdown on cartilage matrix and protease expression

As chondrosarcoma growth and spread requires both production of new cartilage-type matrix and the ability of the cells to migrate through the specialised matrix, we assessed whether CSPG4/NG2 knock down influenced expression of cartilage matrix genes and proteolytic enzymes known to be involved in cartilage breakdown. There was no difference in expression of aggrecan or type II collagen, the major cartilage matrix molecules (not shown). In contrast CSPG4/NG2 knockdown had significant effects on protease gene expression (Fig 5a). Expression of both *MMP3* and *ADAMTS4* genes were decreased in B3 (CSPG4/NG2 –ve) cells in contrast to the CSPG4/NG2 +ve JJ012 and non-target transfected control cells. Conversely, *MMP13* gene expression was increased in the B3 cells. *ADAMTS5* expression was minimal in all three cell types.

CSPG4/NG2 expression is associated with relative resistance to doxorubicin but not docetaxel induced cell death.

CSPG4/NG2 expression is associated with increased resistance to standard chemotherapy and radiotherapy in a number of tumour types. To investigate whether CSPG4/NG2 expression might contribute to chemoresistance in chondrosarcomas, viability and apoptosis of CSPG4/NG2 positive (JJ012) and negative (B3) chondrosarcoma cells was assessed following treatment with doxorubicin or doxotaxel. Initial studies identified an IC_{50} of 0.3µM and 10nm for doxorubicin and docetaxel respectively when applied to monolayer cultures of JJ012 for 48 hours (not shown) and these concentrations were used for subsequent studies. Assessment of annexin V expression by flow cytometry indicated that apoptosis was greater in B3 cells treated for 48 hours with 0.3µM doxorubicin in comparison to JJ012 cells and non-target control chondrosarcoma cells (Fig 5b). In contrast there was no significant difference in apoptosis between JJ012 and B3 cells after 48 hours treatment with 10nM docetaxel (not shown).

Discussion

In this study, we have shown that knockdown of the multifunctional transmembrane proteoglycan CSPG4/NG2 in the JJ012 chondrosarcoma cell line is associated with a decrease in cell proliferation and migration, whilst cell spreading on extracellular matrices was increased. CSPG4/NG2 knock down was also associated with changes in expression of *MMP3*, *MMP13* and *ADAMTS4* although no significant differences were identified between the CSPG4/NG2 positive and negative cells in a transwell invasion assay. Importantly CSPG4/NG2 expression also appeared to provide a degree of protection against doxorubicin induced apoptosis.

CSPG4/NG2 is recognised to be expressed by chondrocytes during cartilaginous bone formation, in articular cartilage and by chondrosarcomas (Fukushi*, et al.*, 2003, Schwab*, et al.*, 2009). The function of CSPG4/NG2 in cartilage has not been widely studied and is not entirely clear. In adult articular cartilage, CSPG4/NG2 appears to be important in the regulation of integrin dependent adhesion of chondrocytes to type VI collagen(Midwood and Salter, 2001), but it is likely that this multifunctional proteoglycan will have a number of roles similar to that identified in other cell types and tissues in which it has been identified.

Over the last decade or so, there has been significant advances in our knowledge of the structure and function of CSPG4/NG2. Structurally, CSPG4/NG2 consists of a large extracellular domain and a short 76 amino acid cytoplasmic domain, separated by a 25 amino acid transmembrane domain (Nishiyama*, et al.*, 1991). The extracellular domain includes sites for binding to a range extracellular matrix proteins (type V and type VI collagens), growth factors (FGF-2 and PDGFaa), integrins, matrix metalloproteinases (MMPs) and lectins. Cleavage of CSPG4/NG2at membrane proximal sites of the extracellular domain results in the release of fragments that can stimulate endothelial cell migration

by interacting with galectin 3 and $\alpha 3\beta 1$ integrin expressed by endothelial cells (Fukushi*, et al.*, 2004, Wen*, et al.*, 2006). The intracellular domain mediates attachment to scaffolding proteins linking CSPG4/NG2 to intracellular signalling pathways and to the actin cytoskeleton(Chatterjee*, et al.*, 2008) whilst phosphorylation of Thr2256 and Thr2314 within the intracellular domain has been found to be critical to balance between cell motility and cell proliferation (Makagiansar*, et al.*, 2004, Makagiansar*, et al.*, 2007, Wang, et al., 2010)

As a consequence of its wide ranging activity in regulating cell function CSPG4/NG2has a number of predicted roles in tumourigenesis such as increased proliferation, migration, adhesion, invasion, metastasis and chemoresistance (Wang *et al.,* 2010 Nicolosi*, et al.*, 2015). In the current study, we have identified potential roles for this molecule in chondrosarcoma cell proliferation, migration and resistance to chemotherapy. CSPG4/NG2 functions predominantly as a co-receptor presenting growth factors to their cognate receptors and through interactions with extracellular matrix molecules such as collagens and fibronectin CSPG4/NG2 is linked to integrin-regulated signalling pathways. The decrease in proliferation and migration seen with CSPG4/NG2 knockdown in the current study is likely to be a combination of disruption of this co-receptor activity. Previous studies, in other cell types, have shown that CSPG4/NG2 can bind to FGF-2 and PDGFaa and potentiate the mitogenic effect of the growth factors through presentation of ligands or by acting as a co-receptor (Grako*, et al.*, 1999). Similarly, NG2-negative pericytes show significantly less proliferation than NG2-positive cells in response to FGF2 (Goretzki*, et al.*, 1999). In association with integrins, CSPG4/NG2activates FAK and ERK signalling pathways potentiating proliferation (Yang*, et al.*, 2004). CSPG4/NG2also plays a major role in migratory behaviour of cells. Targeting CSPG4/NG2reduces cell attachment and spreading (Bumol*, et al.*, 1984) by interfering with integrin and non-integrin dependent cell adhesion to matrix components such as collagens II, V and VI, laminin and fibronectin. By regulating cellular attachment to these extracellular components CSPG4/NG2 can potentiate cell migration (Burg*, et al.*, 1996, Tillet*, et al.*, 1997). Indeed CSPG4/NG2 co-localisation with integrins and small GTPases in filopodia at the

leading edge of motile cells (Lin*, et al.*, 1996a, Lin*, et al.*, 1996b) may help to regulate the internal architecture of the cell in response to signals from its environment aiding migration.

In addition to functioning as a co-receptor and transducing signals from the environment, CSPG4/NG2 has also been shown to actively modify the ECM through enhancing tissue breakdown. Studies from melanoma have demonstrated that mediates melanoma cell invasion through collagen I by activation of MT1-MMP and MMP2 (Iida*, et al.*, 2007). Thus the observations that knockdown of CSPG4/NG2 in the chondrosarcoma cell line in the current study results in decreased expression of *MMP3* and *ADAMTS4* may be particularly relevant for chondrosarcoma cell invasion although, in the current study, we were unable to demonstrate a significant difference in cell invasion in a transwell assay. ADAMTS4 has a major role in cartilage breakdown in degenerative conditions such as osteoarthritis (Song*, et al.*, 2007) whilst MMP3, in addition to having direct proteolytic effects on cartilage matrix molecules, activates other MMPs such as MMP-9(Vempati*, et al.*, 2007) a type IV collagenase that has major roles in the invasion and metastasis of a range of cancer types (Stetler-Stevenson, 1990). In contrast to ADAMTS4 and MMP3, expression of MMP13 was increased with CSGP4/NG2 knock down. The reasons for this difference is not clear but a recent study has highlighted a relationship between NG2 and MMP13 and regulation of anoikis (Joo*, et al.*, 2014) raising the possibility of a feedback loop between cell surface or extracellular CSPG4/NG2 levels and MMP13 production which has been lost in the B3 chondrosarcoma (NG2/CSPG4 knock down) cells .

Knockdown of CSPG4/NG2 in our *in vitro* chondrosarcoma cell line increased the efficacy of doxorubicin inducing apoptotic cell death. Similar observations in which CSPG4/NG2 expression promotes chemoresistance have been made in other tumour types and appears to be mediated by CSPG4/NG2 association with integrin-induced activation of PI3K/Akt signalling, a critical component of cell survival signalling pathways(Chekenya*, et al.*, 2008). CSPG4/NG2expression has also been linked to the up-regulation of drug transporter proteins in mixed-lineage leukemia (MLL) cells (Nicolosi, et al., 2015). A major problem with current management of chondrosarcoma treatment is resistance to

both standard chemotherapy and radiotherapy. Targeting CSPG4/NG2may have the potential to enhance chemotherapy effects *in vivo*. Significantly, as a cell surface molecule, CSPG4/NG2 is readily amenable to small molecule and antibody-based immunotherapy (Nicolosi, et al., 2015) Early clinical trials with immunotherapy in melanoma and brain tumours have shown benefit with patients showing significantly increased survival and abolishment of metastasis (Mittelman*, et al.*, 1995, Mittelman*, et al.*, 1994, Mittelman*, et al.*, 1992). The results of the current study indicate that CSPG4/NG2 has roles in regulating chondrosarcoma cell function in relation to growth, spread and resistance to chemotherapy and that anti-CSPG4/NG2 therapies may have potential in the treatment of surgically irresectable chondrosarcoma.

Conflicts of Interest Statement

The authors declare no conflicts of interest.

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Figure Legends.

Figure 1. CSPG4/NG2 expression in the JJ012 chondrosarcoma cell line. (a): Western blot of protein extracted from the JJ012 chondrosarcoma cell line (lanes 2 and 3) and chondrocytes retrieved from human knee joint osteoarthritic cartilage (lanes 4 and 5) which were untreated (lanes 3 and 5) or pretreated with chondroitinase ABC (lanes 2 and 4). Lane 1 contains molecular weight markers. (b) Immunofluorescent labelling of CSPG4/NG2 in JJ012 and osteoarthritic (OA) chondrocytes. Control = non-immune serum negative control. (c) CSPG4/NG2 expression in a human chondrosarcoma sample. Main figure bar = 500 μm, insert bar = 125 μm

Figure 2. Knock-down of CSPG4/NG2 in the JJ012 cell line. Upper panel: relative RNA expression in the JJ012 cell line and in three clones (D1, D2 and B3) following shRNA lentiviral transduction. Lower panel: Western blot of extracted protein from the JJ012, D1, B3 and GFP transduced control.

Figure 3. Effect of CSPG4/NG2 knock-down on (a) chondrosarcoma cell proliferation (upper panel) and apoptosis lower panel and (b) chondrosarcoma cell adhesion (upper panel) and spreading (lower panel).

Figure 4. Effect of CSPG4/NG2 knock-down on (a) chondrosarcoma cell migration. Photomicrographs of monolayer cultures immediately and 10 hours following wounding. The lower panel shows quantitative measurement of wound closure. (b) Chondrosarcoma cell invasion in a Matrigel transwell system using serum or EGF as chemoattractants.

Figure 5. . Effect of CSPG4/NG2 knock-down on (a) cartilage MMP and aggrecanase expression. NT = non target control, B3 = CSPG4/NG2 knock down cells, JJ = JJ012 parent chondrosarcoma cell line. (b) Response to doxorubicin treatment. CSPG4/NG2 knock-down is associated with relative resistance to doxorubicin. Cells were treated with 0.3μM doxorubicin for 48 hours and the number of apoptotic cells counted.

Figure 1.

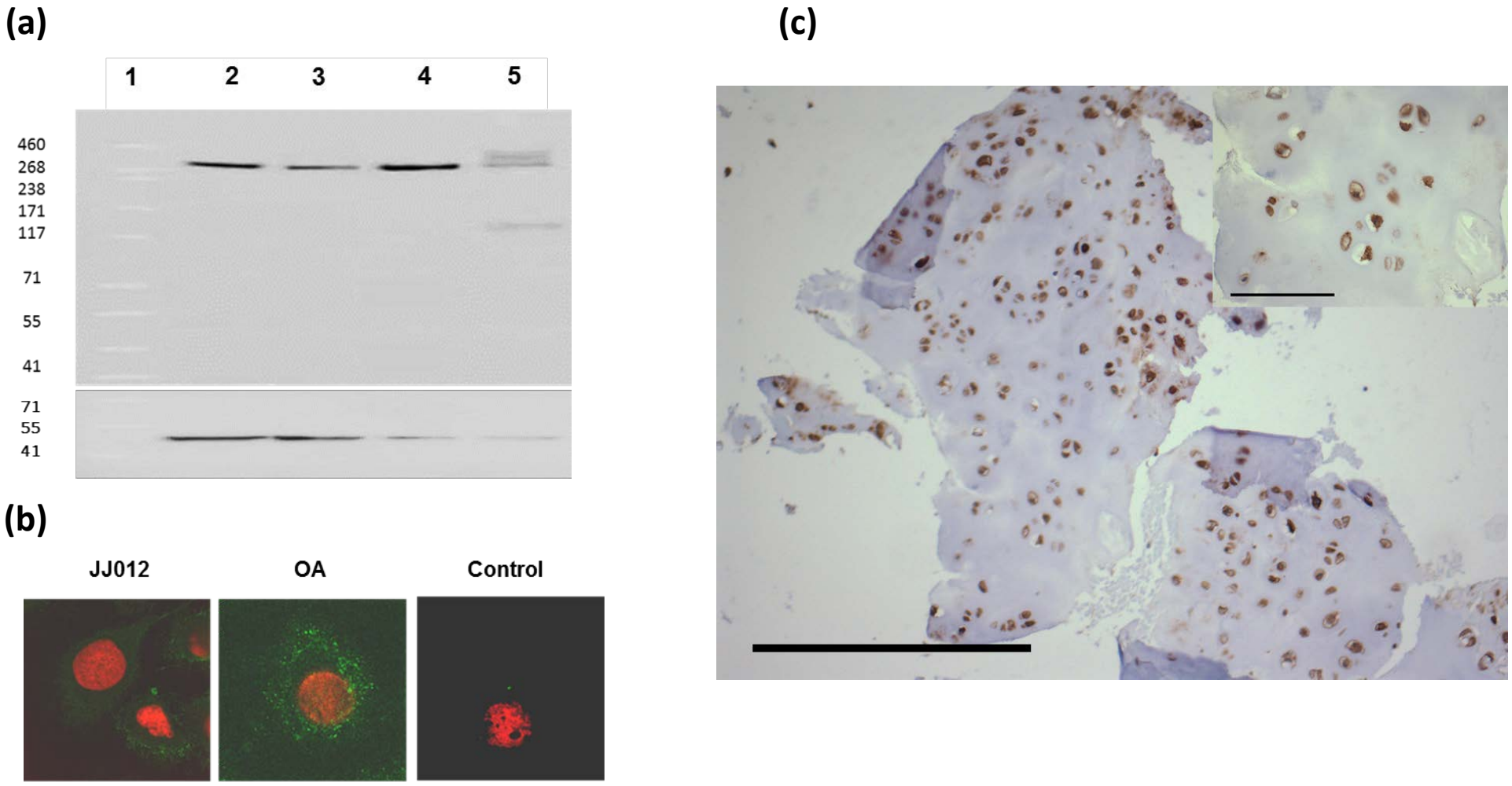
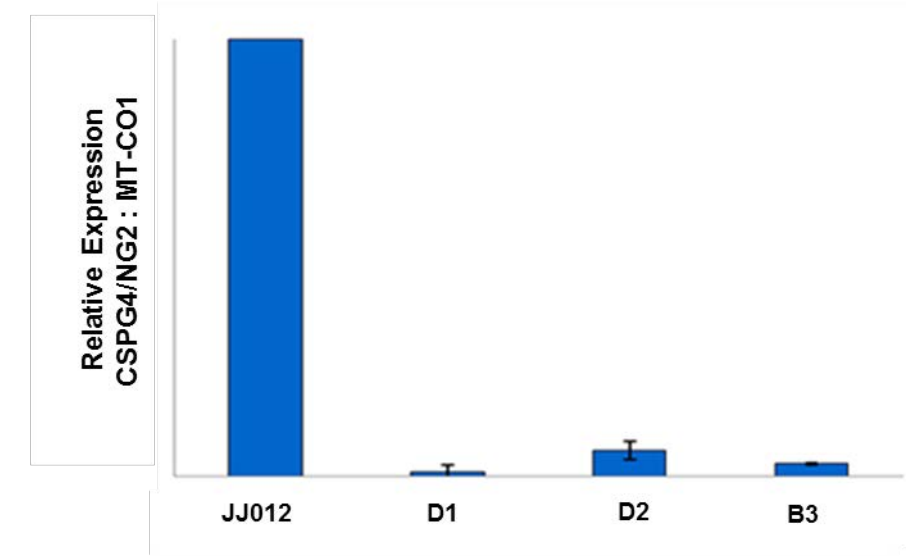


Figure 2.


Relative Expression
CSPG4/NG2 : MT-CO1
JJ012
D1
D2
B3

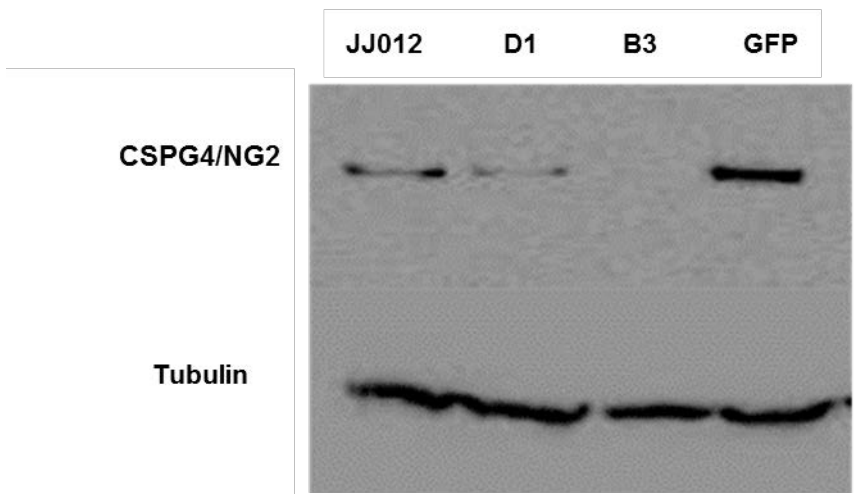
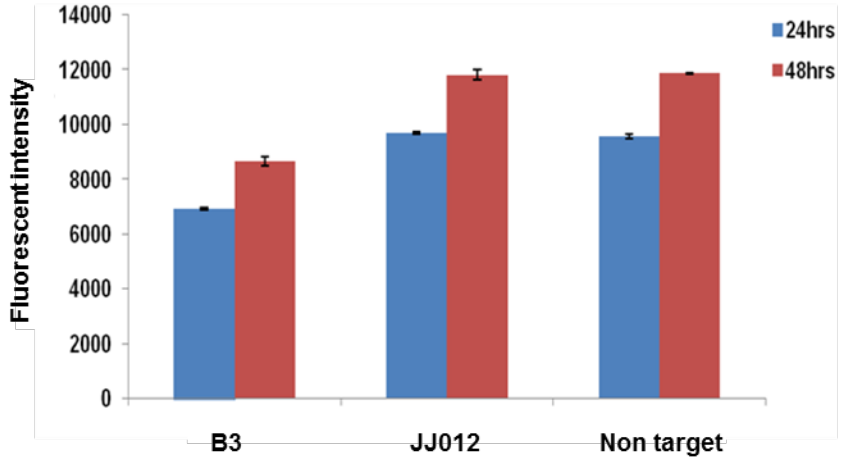
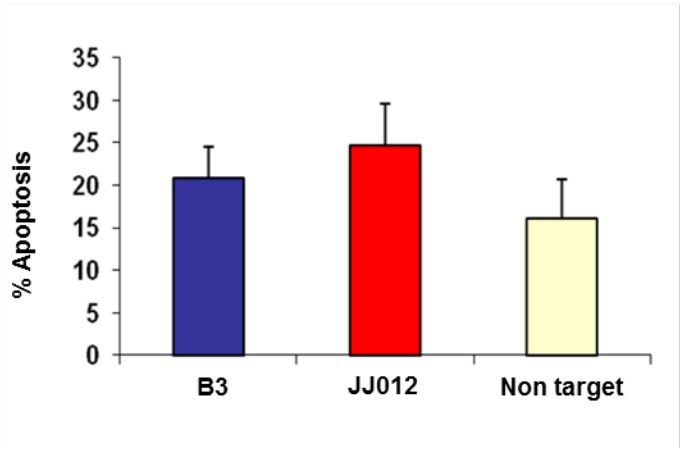

JJ012
D1
B3
GFP
CSPG4/NG2
Tubulin

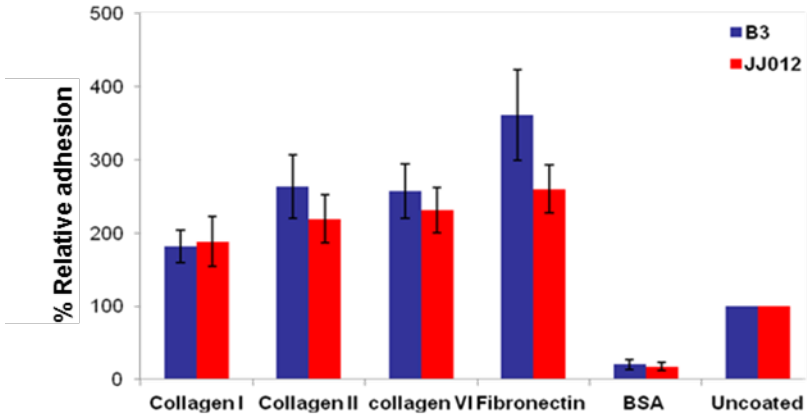
Figure 3.

(a)


Fluorescent Intensity
14000
12000
10000
8000
6000
4000
2000
0
24hrs
48hrs
B3
JJ012
Non target


% Apoptosis
35
30
25
20
15
10
5
0
B3
JJ012
Non target

(b)


% Relative adhesion
500
400
300
200
100
0
B3
JJ012
Collagen I
Collagen II
collagen VI
Fibronectin
BSA
Uncoated

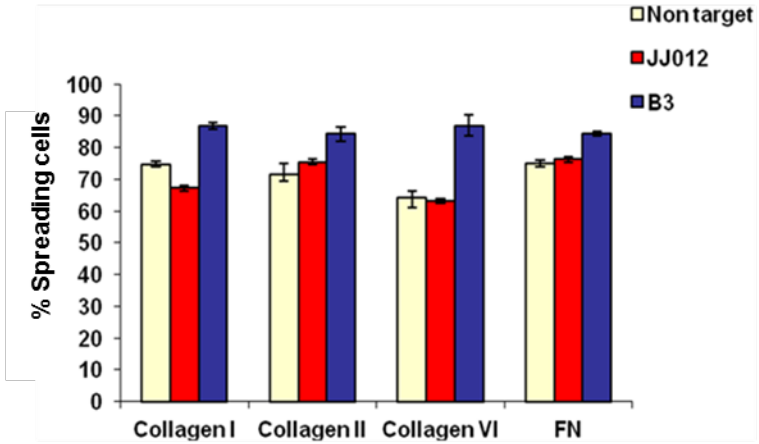
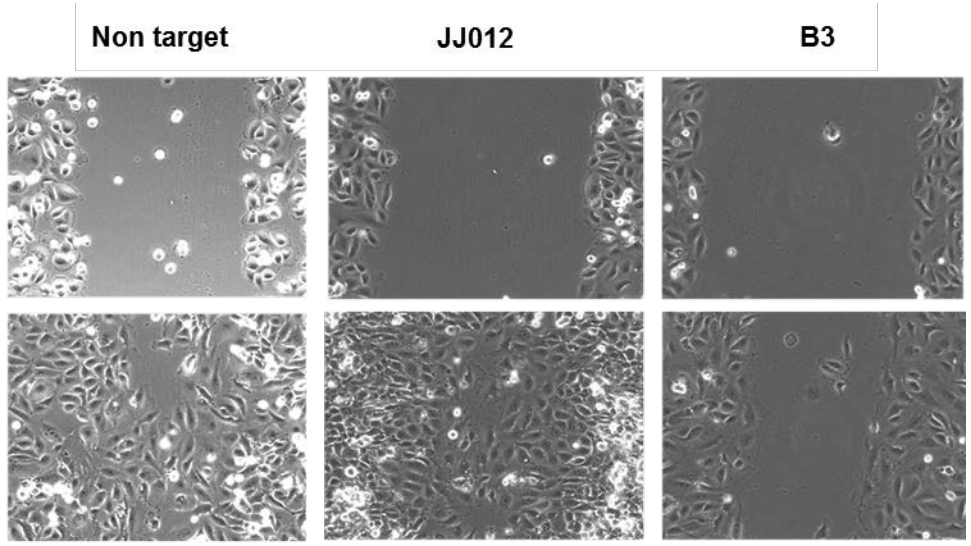
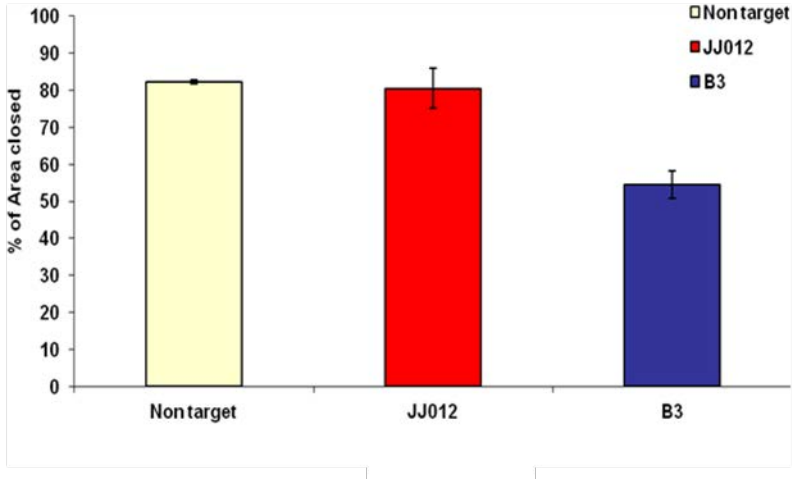

% Spreading cells
100
90
80
70
60
50
40
30
20
10
0
Non target
JJ012
B3
Collagen I
Collagen II
Collagen VI
FN

Figure 4.

(a)


Non target
JJ012
B3


% of Area closed
100
90
80
70
60
50
40
30
20
10
0
Non target
JJ012
B3
Non target
JJ012
B3

(b)

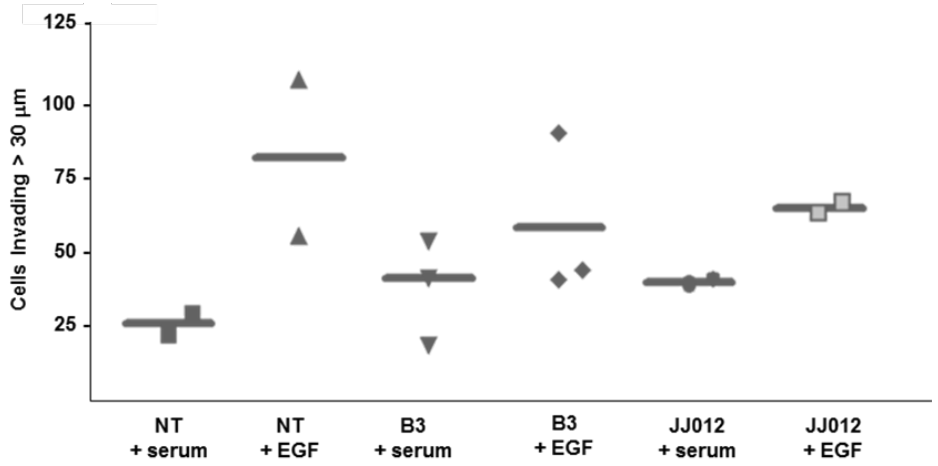
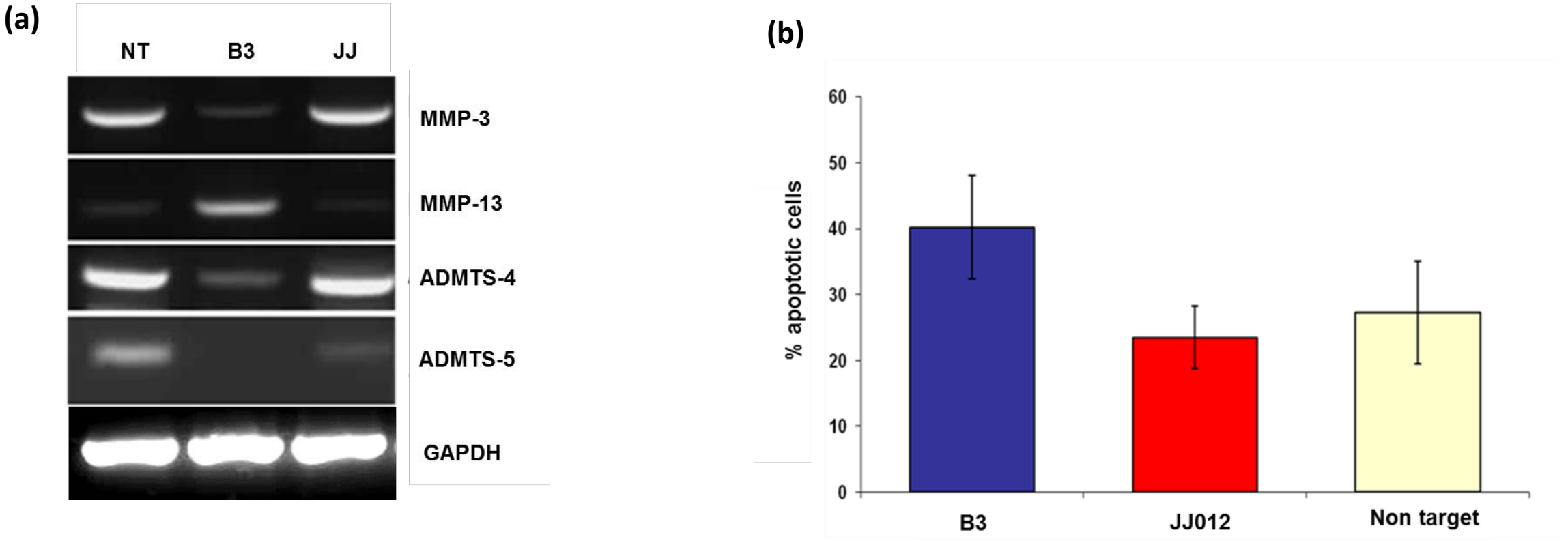

Cells Invading > 30 µm
125
100
75
50
25
NT + serum
NT + EGF
B3 + serum
B3 + EGF
JJ012 + serum
JJ012 + EGF

Figure 5.


(a)
NT
B3
JJ
MMP-3
MMP-13
ADMTS-4
ADMTS-5
GAPDH
(b)
% apoptotic cells
60
50
40
30
20
10
0
B3
JJ012
Non target