CCN3 suppresses mitogenic signalling and reinstates growth control mechanisms in Chronic Myeloid Leukaemia

Lynn McCallum · Wanhua Lu · Susan Price ·
Noureddine Lazar · Bernard Perbal ·
Alexandra E. Irvine

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Abstract CCN3, a tumour suppressor gene, is down-regulated as a result of BCR-ABL tyrosine kinase activity in Chronic Myeloid Leukaemia (CML). We have established a stable CCN3 expression model in the human K562 CML cell line and have further validated the role for CCN3 in the leukaemogenic process. K562 cells stably transfected with CCN3 (K562/CCN3; 2.25×10^6 copies per 50 ng cDNA) demonstrated over 50% reduction in cell growth in comparison to cells stably transfected with empty vector (K562/control; $p=0.005$). K562/CCN3 cells had reduced colony formation capacity (reduced by 29.7%, $p=0.03$) and reduced mitogenic signalling in comparison to K562/control cells (reduced by 29.5% ($p=0.002$) and 37.4% ($p=0.017$) for phosphorylation levels of ERK and AKT respectively). K562/CCN3 cells showed an accumulation of events within the subG₀ phase of the cell cycle and increased apoptosis was confirmed by a three-fold increase in annexin V binding

($p<0.05$). K562/CCN3 cells exposed to Imatinib (1 μ M and 5 μ M) showed an increase in events within the subG₀ phase of cell cycle over 96 h and mirrored the enhanced cell kill demonstrated by Annexin staining. Wild type K562 cells treated with recombinant human Ccn3 (10 nM) in combination with Imatinib (5 μ M) also displayed enhanced cell kill ($p=0.008$). K562/CCN3 cells displayed increased adhesion to matrigel™ (2.92 ± 0.52 fold increase compared to K562/control) which was commensurate with increased expression of the alpha 6 and beta 4 integrins (6.53 ± 0.47 and 1.94 ± 0.07 fold increase in gene expression respectively ($n=3$, $p<0.05$)). CCN3 restores cellular growth regulatory properties that are absent in CML and sensitises CML cells to imatinib induced apoptosis. CCN3 may provide novel avenues for the development of alternate therapeutic strategies.

Keywords CCN3 · BCR-ABL · Imatinib · ERK · AKT · Integrin · Signalling

L. McCallum
School of Biomedical and Biological Sciences, Portland Square,
University of Plymouth,
Drake Circus,
Plymouth PL4 8AA, UK

W. Lu · S. Price · A. E. Irvine (✉)
Centre for Cancer Research and Cell Biology,
Queen's University of Belfast,
97 Lisburn Road,
Belfast BT9 7AB, UK
e-mail: s.irvine@qub.ac.uk

N. Lazar
Universite Paris 7D Diderot,
Paris 75005, France

B. Perbal
L'Oreal R&D,
Clark, NJ 07066, USA

Introduction

The CCN family of matricellular proteins were named using the first initial of the founding members CCN1-3; Cyr61, CTGF and Nov. The more recently identified members CCN 4–6 comprise the Wnt induced secreted proteins WISP1-3. CCN proteins share regions with high homology housed within four domains, the insulin-like growth factor binding (IGFBP), Von Willebrand type C (VWC), Thrombospondin type I (TSP-1) and cysteine rich carboxyl terminal (CT) domains.

CCN3 was originally identified as an integration site in an avian nephroblastoma model of Wilm's tumor induced by myeloblastosis associated virus (MAV) (Chevalier et al. 1998).

CCN3 has mostly been associated with tumour suppressor activity; inhibiting cell growth and tumourigenic potential in glioblastoma (Sin et al. 2008, Fu et al. 2004), melanoma (Fukunaga-Kalabis et al. 2006, Fukunaga-Kalabis et al. 2008) and adrenocortical tumours (Martinerie et al. 2001). CCN3 has also been linked with enhanced metastatic potential for some tumours including Ewings Sarcoma (Perbal et al. 2009), osteosarcoma (Perbal et al. 2008) and prostate neoplasia (Maillard et al. 2001).

Our group were the first to identify a role for CCN3 in haematological malignancy showing CCN3 was down-regulated as a result of the BCR-ABL oncogene in CML (McCallum et al. 2006). CML is a clonal disorder of pluripotent haematopoietic stem cells and CML progenitor cells possess a subtle defect in cell maturation (Wong and Witte 2001). Additional phenotypic changes occur in cell proliferation, adhesion and resistance to the induction of apoptosis (Deininger et al. 2000, Jorgensen and Holyoake 2001, Verfaillie et al. 1997). CCN3 expression is low in CML patients at diagnosis and increases to levels similar to that found in normal bone marrow in response to the BCR-ABL tyrosine kinase inhibitor, Imatinib (McCallum et al. 2009). Although Imatinib has revolutionised current therapeutic intervention strategies, resistance to this compound and its second generation derivatives is becoming increasingly clear (Deininger and Holyoake 2005, Heaney and Holyoake 2007). Alternate strategies are required to effectively manage this disease.

We have previously shown, using a transient expression model, that increasing CCN3 expression results in reduced cell growth and enhanced apoptosis of CML cells (McCallum et al. 2006, McCallum et al. 2009). We now report on the establishment of a CCN3 stable expression system in K562 CML cells and characterization of this model. CML cells stably expressing the CCN3 gene (K562/CCN3) show reduced growth capacity as well as reduced mitogenic signalling. Expression of CCN3 in K562 cells sensitises them to undergo increased apoptosis in response to imatinib treatment. Similarly, wild type K562 cells show increased imatinib induced cell kill when treated with recombinant human Ccn3 (rhCcn3).

Materials and methods

Cell culture

K562 cells (DSMZ GmbH, Braunschweig, Germany) were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (Gibco BRL, Paisley, UK). Cells were subcultured twice weekly to maintain a log phase culture of 2×10^5 cells/ml and maintained in a humidified incubator at 37°C in 5% CO₂ in air.

To obtain cells stably expressing CCN3, K562 cells were nucleofected following the manufacturer's instruction using the Cell Line Nucleofector Kit V, program T-16 (Amaxa, Cologne, Germany) with the pCMV82 vector encoding human full-length CCN3 (5 µg) or empty vector (pCsb6+) as a control. Stable transfectants were selected in G418 (Invitrogen, Paisley, UK) at a concentration of 800 µg/ml. Single cell colonies were produced by serial dilution in the presence of G418, then allowed to grow and subsequently tested for CCN3 expression by RQ-PCR and Western blotting.

Where appropriate, cells were incubated with Imatinib (1 µM or 5 µM, Novartis Pharmaceuticals Corporation, New Jersey, USA) and recombinant human Ccn3 (10 nM, Peprotech EC Ltd, London, UK).

RQ-PCR

RNA was extracted using Trizol (Invitrogen, UK) according to manufacturers' instructions. RNA (2 µg) was reverse transcribed using the mMLV reverse transcriptase after DNase I treatment (both Invitrogen). RQ-PCR was performed using an ABI PRISM 7500 Sequence Detector which exploits TaqMan probe based chemistry (Applied Biosystems, Warrington, UK). Primer and probe sets for CCN3, Integrins alpha 6, beta 1 and beta 4 were designed against Genbank published sequences in association with Primer Express (Applied Biosystems). CCN3 primers and probe set were used as previously described (McCallum et al. 2006) having the 5' reporter 6-carboxyfluorescein (FAM) and the 3' quencher 6-carboxy-tetramethylrhodamine (TAMRA). The amplification reactions (12.5 µL) contained 50 ng cDNA equivalents (or control), 1×Taqman universal PCR master mix, final concentrations of 5 mM MgCl₂, 0.2 mM deoxyadenosine deoxycytosine deoxyguanosine triphosphate (dATP/ dCTP/ dGTP), 0.4 mM deoxyuridine triphosphate (dUTP), 0.125 U AmpliTaq Gold, 2 µM primers (forward and reverse) and 200 nM TaqMan probe. Amplifications were performed following an initial 20 s incubation at 50°C to allow uracil-N-glycosylase (UNG) to destroy any contaminating RNA, followed by treatment at 95°C for 10 min to inactivate the UNG enzyme and activate the AmpliTaq Gold DNA polymerase. This was followed by 40 cycles of denaturing at 95°C for 15 s and annealing/extension at 60°C for 1 min. Data were collected and analysed with Sequence Detector v1.6.3 software (Applied Biosystems). Relative quantitative (RQ-PCR) data was calculated based on the $\delta\delta C_T$ method (Ginzinger 2002). Amplification of integrin specific cDNA was performed using the FastStart universal SYBR Green master mix (Roche Applied Science, Mannheim, Germany) with primers (300 nM) which amplified integrin specific single product by melt curve.

Cell growth assays

Cells were seeded in triplicate in a 24-well plate at a density of 1×10^5 cells per ml. Cell number and viability was counted every 24 h using a haemocytometer and trypan blue exclusion (Gibco BRL, Paisley, UK). Results were plotted for live cells against time to generate a growth curve. Alternatively CyQuant® (Invitrogen, UK), a fluorescent dye which binds DNA and is independent of cell metabolic processes, was used to assess cell number in the presence or absence of Matrigel™ as described previously (Lu et al. 2009).

Colony formation assays

Colony forming capacity was determined by plating cells (1.0×10^3) in triplicate in methylcellulose cultures (Stem Cell Technologies, Vancouver, BC, Canada) as previously described (McCallum, Price et al. 2006). Cells were allowed to grow for 7 days and then counted using an inverted microscope (Olympus, Tokyo, Japan (magnification $\times 40$)).

Western blotting

Protein was extracted from cells by suspending in RIPA buffer ($1 \times$ PBS, 1% Nonidet NP-40, 0.1% SDS) containing a cocktail of protease inhibitors (Complete Mini Cocktail, Roche Diagnostics Ltd, Lewes, UK) at a concentration of 10^7 cells / mL and lysing for 10 min on ice. Samples were sonicated for 10 s to ensure complete lysis, centrifuged at 10,000 g at 4°C for 10 min and the supernatant removed for analysis. Total protein content was determined by the Bradford protein method using the BCA protein assay kit (Pierce, Cramlington, UK). Protein (30 µg) was loaded onto a pre-cast Bis-Tris polyacrylamide gel (10%) using the Novex mini gel system (Invitrogen, Paisley, UK) and subsequently transferred to a PVDF membrane. Ccn3 expression was detected using an antibody raised against the C terminal of Ccn3 (NH5, courtesy of B.Perbal). Immunoblots were visualised by enhanced chemiluminescence (Supersignal West Pico and Supersignal West Dura substrates; Pierce, Rockford, IL, USA).

Antibodies used for Western blotting were as follows: total ERK 1/2 (Santa Cruz Biotechnology, Santa Cruz, CA; USA 1:500), phospho-ERK 1/2, total Akt, phospho-Akt and pan actin (all Cell Signalling Technology, Danvers, MA, USA; 1:1000).

Flow cytometry

Cells (5×10^5) were fixed in ice cold 70% ethanol for flow cytometric analysis of cell cycle status. Fixed cells were resuspended in PBS and incubated in RNase A (100 mg/ml, Qiagen) and propidium iodide (40 mg/ml, Calbiochem) for

30 min at 37°C. Samples were then analyzed on a Coulter Epics Elite Cell Sorter (Beckman Coulter, High Wycombe, United Kingdom). Data were collected on the basis of peak signal versus integral signal to exclude doublets. Analysis was performed on 20,000 events using winMDI software (<http://facs.scripps.edu/software.html>) and data were generated and then ranked from a common gate in the subG₀ area.

The ApoScreen Annexin V Apoptosis Kit (BD Biosciences, Bedford, MA, USA) was used to confirm apoptosis. According to the manufacturer's instructions cells (1×10^5) were harvested and washed twice in cold PBS, then resuspended in 100 µl of $1 \times$ binding buffer with 5 µl each of annexin V-PE and 7-AAD. After gently mixing, the reactions were wrapped in foil and incubated at room temperature for 15 min. Samples were then analyzed within 1 h on a Coulter Epics Elite Cell Sorter (Beckman Coulter, High Wycombe, UK). Analysis was performed on 30,000 events using winMDI software, and data were generated and then ranked from a gate in the annexin V positive area.

Cell adhesion assays

The adherent ability of stable CCN3-expressing K562 cells to Matrigel™ matrix was examined by using BioCoat Matrigel™ 96-well plates (BD Biosciences, Bedford, USA) which were coated with a thin layer of Matrigel™ in each well. The plate was rehydrated by adding 150 µl of warm culture medium to each well and incubated at 37°C for 30 min. A cell suspension (100 µl) containing 5×10^4 cells was seeded after carefully removing the rehydration medium. The plate was incubated at 37°C for 24 h to allow cells to adhere to the Matrigel™ layer. Non-adherent cells were removed by gently washing the wells 5 times with room temperature PBS. The number of adherent cells was evaluated by using CyQuant Cell Proliferation Assay Kit (Invitrogen, Paisley, UK) as previously described (Lu et al. 2009).

Cell adhesion was tested on tissue culture plates coated with cell lysate from stably expressing CCN3 cells or control cell lysate. In brief, a 96-well flat bottom plate was coated at 4°C overnight with 50 µl cell lysate prepared from K562/CCN3 or K562/control cells. To block any remaining protein binding sites on the plate, 150 µl of 1.0% BSA in PBS per well was added and incubated at room temperature for 30 min. For neutralization experiments, 150 µl of 1.0% BSA containing 1 µl Ccn3 antibody (anti-K19M or anti-NH5 antibody) was used. In each assay, a background control was set up by coating the wells with 1.0% BSA alone. Immediately prior to adding cells, the wells were washed gently three times with 150 µl room temperature PBS by dispensing PBS against the sides of the wells. Cell suspension (100 µl) containing 5×10^4 wild type K562 cells was added to each well and incubated at 37°C overnight. Wells were then washed gently 5 times with room

Fig. 1 CCN3 causes growth inhibition of CML cells. **a** K562 CML cells were transfected to stably express a full length CCN3 construct (K562/CCN3) or empty vector only (K562/control). **(i)** Western blot analysis was performed on lysates from K562/CCN3 and K562/control cells using an antibody raised to the carboxyl terminal of CCN3 (NH5). **(ii)** RQ-PCR was performed on RNA extracted from K562/CCN3 and K562/control cells to detect the number of CCN3 transcripts per 50 ng of cDNA. **b** Cell growth was monitored for both K562/CCN3 and K562/control cells by assessing cell number daily

over 6 days **(i)** and colony formation capacity in methyl cellulose culture on day 7 **(ii)**. **c** Western blot analysis was performed on lysates from K562/CCN3 and K562/control cells to examine levels of **(i)** ERK phosphorylation in comparison to levels of total ERK, **(iii)** Akt phosphorylation in comparison to levels of total Akt. Pan actin was used as a control gene to ensure equivalent protein loading. **(ii)** and **(iv)** refer to optical densitometry performed on the blots and phosphorylation data are shown as arbitrary units in comparison to K562/control ($n=3$; * $p<0.05$)

temperature PBS to remove non-adherent cells. Detection of the remaining adherent cells was performed using the CyQuant Cell Proliferation Assay (Invitrogen, Paisley, UK).

Results

CCN3 reduces cell growth and mitogenic signalling

To investigate the role of CCN3 in CML, human K562 CML cells were stably transfected with vector alone (K562/control) or vector containing CCN3 construct (K562/CCN3). Cells were then compared for growth characteristics, colony forming capacity and effect on the ERK/AKT mitogenic signalling pathways. Western blot analysis was performed to detect levels of CCN3 expression in both K562/control and K562/CCN3 cells. Ccn3 protein was below the level of detection in the K562/control cells, whilst Ccn3 was clearly expressed in the K562/CCN3 cells at 48 kDa (Fig. 1a (i)). Real-time PCR was performed to quantitate the level of CCN3 expression using the CCN3 vector to generate transcript copy numbers. CCN3 transcript numbers were again barely detectable in the K562/control cells (3.4 ± 1.5 copies per 50 ng cDNA) compared to K562/CCN3 cells with over 2 million copies (2.25×10^6 copies per 50 ng cDNA) (Fig. 1a (ii)). Stable increased expression of CCN3 in K562 cells showed reduced growth of K562/CCN3 cells compared to K562/control cells (Fig. 1b (i)). By Day 5, K562/CCN3 cells showed a 48% reduction in cell growth in comparison to K562/control cells ($p=0.005$, $n=3$). Further evaluation by colony forming assays performed in methyl cellulose culture showed that by Day 7, K562/CCN3 cells had reduced colony formation capacity (Fig. 1b (ii)); reduced by 29.7% on Day 7 compared to K562/control cells ($p=0.003$, $n=3$). We then looked at mitogenic signalling pathways associated with cell proliferation and survival. Western blotting was performed on lysates from both K562/CCN3 and K562/control cells. Stable expression of Ccn3 reduced the level of ERK1/2 phosphorylation (Fig. 1c (i)) whilst levels of ERK1/2 protein remained constant and pan actin levels were comparable. Similarly, K562/CCN3 cells showed reduced levels of Akt phosphorylation (Fig. 1c (iii)) whilst total Akt and pan actin levels remained constant. Optical densitometry was performed on the western blots and graphs show magnitude of phosphorylation for ERK (Fig. 1c (ii)) and Akt (Fig. 1c (iv)) for K562/CCN3 and K562/control cells respectively ($n=3$, $p<0.05$).

CCN3 expression augments the induction of apoptosis

Since CCN3 stable expression reduced K562 cell growth (Fig. 1), we then investigated if the growth inhibition was

a result of increased apoptosis. Cell cycle analysis showed an increase in the accumulation of events within the subG₀ phase of cell cycle in K562/CCN3 cells compared to K562/control cells (Fig. 2a (i)). CCN3 significantly increased the number of events in the subG₀ phase of cell cycle ($7.3\% \pm 3.0$ for K562/control vs $11.4\% \pm 2.8$ for K562/CCN3 cells; $p=0.04$, $n=3$) (Fig. 2a (ii)). Changes in the proportion of events within other phases of the cell cycle did not appear to be significantly altered. To ensure these events were a true measure of apoptosis, we performed the Annexin V assay which measures the amount of Annexin V bound to phosphatidyl serine on the cell surface, which signifies apoptosis or cell death. K562/CCN3 cells had a three fold increase in Annexin V binding in comparison to control cells ($23.9\% \pm 5.5$ for CCN3 and $8.4\% \pm 3.4$ for control cells respectively; $n=3$, $p<0.05$) (Fig. 2a (iii)). To then determine if CCN3 expression could enhance the magnitude of induction of apoptosis in response to a BCR-ABL tyrosine kinase inhibitor, we subjected K562/CCN3 and K562/control cells to treatment with imatinib ($1 \mu\text{M}$ and $5 \mu\text{M}$) for 48 h. CCN3 expression increased the number of events within the subG₀ phase of cell cycle in response to imatinib treatment. Figure 2b (i) shows the increased number of subG₀ events for K562/CCN3 cells above K562/control, in response to $1 \mu\text{M}$ (13.7 ± 4.6 ; $p=0.04$) and $5 \mu\text{M}$ (16.2 ± 3.3 ; $p=0.001$) imatinib treatment ($n=3$). To then investigate if CCN3 could invoke a cumulative effect in response to imatinib treatment we investigated the magnitude of subG₀ events over 96 h. K562/CCN3 cells showed increased levels of SubG₀ events at 48, 72 and 96 h (16.8 ± 6.7 , 22.5 ± 0.6 and 22.4 ± 3.9 respectively ($n=3$; $p<0.05$) in comparison to K562/control cells treated with imatinib ($5 \mu\text{M}$) (Fig. 2b (ii)). To then determine if the increase in apoptosis was mirrored by a reduction in cell number we performed the CyQuant™ assay (Invitrogen, Paisley, UK). K562/CCN3 cells showed enhanced imatinib induced growth inhibition at $1 \mu\text{M}$ ($44.9\% \pm 0.009$ for CCN3/K562 and $15.3\% \pm 0.005$ for K562/control cells, $n=3$) and $5 \mu\text{M}$ concentrations ($58.3\% \pm 0.030$ for K562/CCN3 and $42.2\% \pm 0.06$ for K562/control cells, $n=3$) at 48 h (Fig. 2b (iii)). To then determine if recombinant human Ccn3 (rhCcn3) could produce a similar effect to CCN3 expression we treated wild type K562 cells with rhCcn3 (10 nM) alone and in combination with imatinib ($5 \mu\text{M}$). Cell number was measured by CyQuant™ assay and results showed that rhCcn3 alone did not alter cell number at 24 h ($103.5\% \pm 0.03$) compared to control (untreated cells). Imatinib treatment reduced cell number by $19.9\% \pm 0.09$ compared to control whilst treatment with rhCcn3 enhanced the imatinib induced cell kill ($28.2\% \pm 0.07$; $n=3$, $p=0.008$) (Fig. 2b (iv)).

Fig. 2 CCN3 increases the induction of apoptosis in CML cells. **a** CCN3 expressing cells (K562/CCN3) and K562/control cells were investigated for induction of apoptosis. **(i)** Flow cytometry was performed on cells stained with propidium iodide and data generated were ranked from a common gate within the subG₀ area of cell cycle. **(ii)** The mean number of ranked events within the common gate in the subG₀ area of cell cycle were plotted graphically as a percentage of overall events for both K562/CCN3 and K562/control cells. **(iii)** Annexin V was used as a substrate to determine the level of phosphatidylserine binding on the cell surface. Data shown are arbitrary values indicating levels of apoptosis. **b** K562/CCN3 and K562/control cells were tested in presence (1 μM or 5 μM) or absence

(control) of imatinib. Flow cytometry was used to assess the number of events within the SubG₀ area of cell cycle for both K562/CCN3 and K562/control cells; the plot shows the increased level of SubG₀ events for K562/CCN3 cells above that of K562/control cells at 48 h **(i)** and at 24 h intervals for 4 days in combination with 5 μM imatinib treatment **(ii)**. The CyQuant™ assay was performed to estimate cell number for K562/CCN3 and K562/control cells in response to imatinib treatment (1 μM and 5 μM) at 48 h **(iii)**. CyQuant™ assay was performed on wild type K562 cells untreated or treated with recombinant human CCN3 (rhCCN3 (10 nM)) in the presence or absence of imatinib (5 μM) for 24 h **(iv)**. * denotes $p < 0.05$

CCN3 increases cell adhesion and integrin expression

To determine if CCN3 induced growth regulation could alter cellular adhesion, we assessed K562/CCN3 and K562/control cells for adhesion to Matrigel™ over 24 h. Increased CCN3 expression produced a three-fold increase in adhesion to Matrigel™ in comparison to K562/control cells ($n=3$, $p=0.049$) (Fig. 3 (i)). To determine if CCN3 was directly responsible for the increased adhesion properties, lysates from K562/CCN3 and K562/control cells were coated onto a 96 well plate and allowed to air dry. Wild type K562 cells were then incubated with the coated plate overnight in the presence and absence of Ccn3 antibody (K19M or NH5). Cells showed a 5-fold increase in adhesion to lysate containing Ccn3 compared to control lysate (Fig. 3 (ii)). In addition, pre-incubation with either K19M or NH5 antibody abrogated the enhanced adhesion of cells to Ccn3 containing cell lysate. To then determine if CCN3 enhanced adhesion could be associated with increased integrin expression we performed RQ-PCR to detect integrin expression levels. K562/CCN3 cells had over 6-fold increase in expression of the alpha 6 integrin and 2-fold increase in expression of the beta 4 integrin subunits in comparison to K562/control cells (Fig. 3 (iii)). There was no difference in expression of the beta 1 integrin between K562/control and K562/CCN3 cells.

Discussion

CML is characterised by expression of the BCR-ABL oncogene. We have previously shown that CCN3 is down-regulated in CML as a direct consequence of BCR-ABL kinase activity; decreased cellular Ccn3 correlated with increased Ccn3 secretion (McCallum et al. 2006).

In this study we have shown that re-expression of CCN3 in K562 CML cells can reverse key aspects of the malignant phenotype. Furthermore, treatment of parental wild type K562 cells with recombinant Ccn3 protein enhanced sensitivity to imatinib although Ccn3 protein used alone was ineffective. This is consistent with CCN3 having tumour suppressor activity within the haematopoietic compartment and is coherent with findings observed for melanoma, glioblastoma and adrenocortical tumours (McCallum and Irvine 2009). Alterations in cell proliferation, differentiation, adhesion and resistance to the induction of apoptosis are hallmarks of the CML phenotype (Verfaillie et al. 1997). Impaired integrin expression and / or integrin signalling not only alters cell adhesion but contributes to the abnormal proliferation of CML progenitors (Verfaillie et al. 1997, Bhatia et al. 1999). We have shown the growth inhibitory effects detected upon increased CCN3 expression in K562 cells are three fold; (i) reducing mitogenic signalling (ii) causing up-regulation of

Fig. 3 CCN3 increases cell adhesion through increased integrin expression. Cells expressing CCN3 (K562/CCN3) and vector only (K562/control) were investigated for adhesion to Matrigel™ basement membrane substrate after 24 h. Adhesion to Matrigel™ was measured using the Cyquant™ assay to estimate cell number and data are expressed as fold change for K562/CCN3 cells (black bars) in comparison to K562/control cells (grey bars) (i). Wild type K562 cells were then allowed to adhere to plastic treated with lysate from K562/control or K562/CCN3 cells overnight in the presence or absence of CCN3 antibody (K19M or NH5). Adhesion was measured using the CyQuant™ assay to estimate cell number and data are shown for K562/CCN3 lysate in comparison to adhesion observed for K562/control cell lysate (ii). RQ-PCR was performed on cDNA from both K562/control and K562/CCN3 expressing cells to detect levels of the alpha 6, beta 4 and beta 1 integrin subunits. (iii) Graph shows the increase in levels of the integrins for K562/CCN3 cells in comparison to K562/control cells (per 50 ng cDNA; $n=3$). * denotes $p<0.05$

integrins and (iii) increasing sensitivity to the induction of apoptosis. Since Ccn3 protein can exert activity as a

secreted, intracellular or transcription factor, it is difficult to delineate which activity or activities are important in restoring growth regulation in CML cells.

The use of recombinant human Ccn3 protein enhanced apoptosis induced by imatinib treatment although the recombinant protein alone did not induce apoptosis in CML cells. This suggests that intracellular Ccn3 activity is required to produce a greater growth inhibitory effect. Since the recombinant protein enhances imatinib induced cell death, this may serve to enhance specificity and selectivity to target CML stem and progenitor cells within the bone marrow microenvironment. These findings imply recombinant Ccn3 effectively ‘primes’ cells for the induction of apoptosis.

In addition, CCN3 up-regulates integrin expression which is consistent with the impaired integrin function effecting cell adhesion (Bhatia et al. 1999) and also contributing to the abnormal proliferation observed in CML progenitors (Verfaillie et al. 1997). The BCR-ABL substrate protein CRKL, crosslinks β integrins as part of the BCR-ABL mitogenic signalling cascade which serves to enhance cell survival through the phospho-inositol-3-kinase (PI3K) / Akt pathway (Sattler and Salgia 1998, Verfaillie 1998). Since we have shown CCN3 reduces Akt phosphorylation and cell survival, it may be conceivable to suggest that CCN3, by causing upregulation of the $\beta 4$ integrin restores sensitivity to the induction of apoptosis which is then exacerbated when combined with imatinib treatment. The role of the $\beta 4$

integrin in haematopoiesis is poorly described, although the role of the $\alpha 6 \beta 4$ integrin is involved in the formation of hemidesmosomes and binding to laminin by epithelial cells (de Pereda et al. 2009, Streuli and Akhtar 2009) which suggests a role in the stabilisation of adhesive processes.

The role of CCN3 causing upregulation of the alpha 6 integrin remains undefined since little is known of its role in haematological terms. It has been proposed that in immunological development the alpha 6 integrin is involved in differentiation stage specific responses to chemokines (Borland and Cushley 2004). This may mirror the subtle defects in cell differentiation associated with the BCR-ABL oncogene (Bhatia et al. 1999). Since the $\beta 1$ integrin is ubiquitously expressed in myelopoiesis and CCN3 is known to bind the $\alpha 6 \beta 1$ integrin (Holbourn et al. 2008) it is reasonable to suggest that CCN3 controls a positive $\alpha 6 \beta 1$ integrin feedback loop, up-regulating integrin expression that it binds to exerting growth regulation and allowing differentiation to proceed in the myeloid compartment.

We propose from our findings that CCN3 reduces mitogenic signalling and up-regulates integrin expression culminating in the reinstallation of growth control mechanisms and induction of apoptosis in CML (Fig. 4). Further investigation of the intricate signalling events associated with cell growth regulation within the myeloid compartment will unravel the role for CCN3 in myelopoiesis and provide alternate avenues for the development of novel targeted therapeutics.

Fig. 4 CCN3 causes upregulation of growth control mechanisms. BCR-ABL stimulates mitogenic signalling pathways including phosphorylation of ERK and Akt enhancing cell proliferation and survival. Increased expression of CCN3 reduces mitogenic signalling either directly or indirectly and may include suppressive integrin signalling to re-instate growth control in CML cells

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References

- Bhatia R, Munthe HA, Verfaillie CM (1999) Role of abnormal integrin-cytoskeletal interactions in impaired beta1 integrin function in chronic myelogenous leukemia hematopoietic progenitors. *Exp Hematol* 27(9):1384–1396
- Borland G, Cushley W (2004) Positioning the immune system: unexpected roles for alpha6-integrins. *Immunology* 111(4):381–383
- Chevalier G, Yeger H, Martinerie C, Laurent M, Alami J, Schofield PN, Perbal B (1998) novH: differential expression in developing kidney and Wilm's tumors. *Am J Pathol* 152(6):1563–1575
- Deininger MW, Holyoake TL (2005) Can we afford to let sleeping dogs lie? *Blood* 105(5):1840–1841
- Deininger MW, Goldman JM, Melo JV (2000) The molecular biology of chronic myeloid leukemia. *Blood* 96(10):3343–3356
- De Pereda JM, Lillo MP, Sonnenberg A (2009) Structural basis of the interaction between integrin alpha6beta4 and plectin at the hemidesmosomes. *EMBO J* 28(8):1180–1190
- Fu CT, Bechberger JF, Ozog MA, Perbal B, Naus CC (2004) CCN3 (NOV) interacts with connexin43 in C6 glioma cells: possible mechanism of connexin-mediated growth suppression. *J Biol Chem* 279(35):36943–36950
- Fukunaga-Kalabis M, Martinez G, Liu ZJ, Kalabis J, Mrass P, Weninger W, Firth SM, Planque N, Perbal B, Herlyn M (2006) CCN3 controls 3D spatial localization of melanocytes in the human skin through DDR1. *J Cell Biol* 175(4):563–569
- Fukunaga-Kalabis M, Martinez G, Telson SM, Liu ZJ, Balint K, Juhasz I, Elder DE, Perbal B, Herlyn M (2008) Downregulation of CCN3 expression as a potential mechanism for melanoma progression. *Oncogene* 27(18):2552–2560
- Ginzinger DG (2002) Gene quantification using real-time quantitative PCR: an emerging technology hits the mainstream. *Exp Hematol* 30(6):503–512
- Heaney NB, Holyoake TL (2007) Therapeutic targets in chronic myeloid leukaemia. *Hematol Oncol* 25(2):66–75
- Holbourn KP, Acharya KR, Perbal B (2008) The CCN family of proteins: structure-function relationships. *Trends Biochem Sci* 33(10):461–473
- Jorgensen HG, Holyoake TL (2001) A comparison of normal and leukemic stem cell biology in Chronic Myeloid Leukemia. *Hematol Oncol* 19(3):89–106
- Lu W, McCallum L, Irvine AE (2009) A rapid and sensitive method for measuring cell adhesion. *J Cell Comm Signal* 3(2):147–149
- Maillard M, Cadot B, Ball RY, Sethia K, Edwards DR, Perbal B, Tatoud R (2001) Differential expression of the ccn3 (nov) proto-oncogene in human prostate cell lines and tissues. *Mol Pathol* 54(4):275–280
- Martinerie C, Gicquel C, Louvel A, Laurent M, Schofield PN, Le Bouc Y (2001) Altered expression of novH is associated with human adrenocortical tumorigenesis. *J Clin Endocrinol Metab* 86(8):3929–3940
- McCallum L, Irvine AE (2009) CCN3—a key regulator of the hematopoietic compartment. *Blood Rev* 23(2):79–85
- McCallum L, Price S, Planque N, Perbal B, Pierce A, Whetton AD, Irvine AE (2006) A novel mechanism for BCR-ABL action: stimulated secretion of CCN3 is involved in growth and differentiation regulation. *Blood* 108(5):1716–1723
- McCallum L, Lu W, Price S, Lazar N, Perbal B, Irvine AE (2009) CCN3: a key growth regulator in Chronic Myeloid Leukaemia. *J Cell Comm Signal* 3(2):115–124
- Perbal B, Zuntini M, Zambelli D, Serra M, Sciandra M, Cantiani L, Lucarelli E, Picci P, Scotlandi K (2008) Prognostic value of CCN3 in osteosarcoma. *Clin Canc Res* 14(3):701–709
- Perbal B, Lazar N, Zambelli D, Lopez-Guerrero JA, Llombart-Bosch A, Scotlandi K, Picci P (2009) Prognostic relevance of CCN3 in Ewing sarcoma. *Hum Pathol* 40(10):1479–1486
- Sattler M, Salgia R (1998) Role of the adapter protein CRKL in signal transduction of normal hematopoietic and BCR/ABL-transformed cells. *Leukemia* 12(5):637–644
- Sin WC, Bechberger JF, Rushlow WJ, Naus CC (2008) Dose-dependent differential upregulation of CCN1/Cyr61 and CCN3/NOV by the gap junction protein Connexin43 in glioma cells. *J Cell Biochem* 103(6):1772–1782
- Streuli CH, Akhtar n (2009) Signal co-operation between integrins and other receptor systems. *Biochem J* 418(3):491–506
- Verfaillie CM (1998) Adhesion receptors as regulators of the hematopoietic process. *Blood* 92(8):2609–2612
- Verfaillie CM, Hurley R, Zhao RC, Prosper F, Delforge M, Bhatia R (1997) Pathophysiology of CML: do defects in integrin function contribute to the premature circulation and massive expansion of the BCR/ABL positive clone? *J Lab Clin Med* 129(6):584–591
- Wong S, Witte ON (2001) Modelling Philadelphia chromosome positive leukemias. *Oncogene* 20(40):5644–5659