ESTABLISHMENT OF MULTIPLE MYELOMA CELL LINES WITH REDUCED EXPRESSION OF HEPATOCYTE GROWTH FACTOR USING RNAI APPROACH

Fouzia Qadir[1], Anders Sudan[2], Asad Jan[3], Shagufta Sultana[4]

[1]Department of Biochemistry North West School of Medicine Peshawar

[2]Department of Clinical and Molecular Medicine. Norwegian University of Science and Technology Trondheim Norway

[3]Institute of Biotechnology and Genetic Engineering(IBGE), University of Agriculture Peshawar

[4]Anatomy Department, Khyber Medical College, Peshawar.

Abstract

Objective: *To establish the gene knockdown system (RNAi) using different Multiple Myeloma Cell lines (MMCL).*

Material and Methods: *RNAi is generally used in biomedical research to study the outcome of knockdown of gene expression. In this research, retroviral-shRNA expression constructs based on micro RNA-adapted short hairpin RNA (sh RNA mir) were designed and constructed. The sequence of the cloned fragments in the vector constructs was determined, and the effect of constructs in MMCLs was examined using qRT-PCR and Western blot analysis.*

Results: *In this work, we established a method for making retroviral-shRNA expression constructs based on micro RNA-adapted short hairpin RNA (sh RNA mir) design. The HGF producing human myeloma cell line JJN-3 transduced with lentiviral HGF-shRNA transduction particles were analyzed for their effect on the HGF gene expression. HGF knockdown MMCL was analyzed both at mRNA level and protein levels. Two out of five clones of myeloma cell line JJN-3 (JJN3-sh3308 and JJN3-sh47137) were transduced with lentiviral HGF–shRNA transduction particles were showing 50-60 % knockdown in their HGF mRNA and protein expression.*

Conclusion: *HGF knockdown cell lines can be used as tissue culture models to investigate the role of HGF protein in the pathology of multiple myeloma. Further, the retroviral-HGF-shRNA expression constructs can study HGF response in various multiple myeloma via gene knockdown studies.*

Keywords: *Multiple myeloma, Hepatocyte growth factor (HGF), Gene knockdown, short hairpin RNA*

INTRODUCTION

Multiple myeloma is a devastating disease that has gained the curiosity of physicians and scientists for decades. Evidence for multiple myeloma has been found in American Indian skeletons from 200 A.D-1300 A.D.[1] However, the well-known early observations about the disease were made in the mid-19th century in a 45 years old London tradesman Mr. Thomas Alexander McBean. Multiple myeloma is the 2nd most frequent cancer among hematological malignancies.[2] The disease causes 0.9% of all cancer-related deaths. The incidence of the disease is higher in developed countries, e.g., Australia, New Zealand, North America, and Europe, while it is lower in Asian countries.[3]

Multiple myeloma is characterized by devastating bone destruction due to the overactivity of

Correspondence:
Dr. Fouzia Qadir
Assistant Professor Department of Biochemistry North West School of Medicine Peshawar.
Email: fouzia.jamil@yahoo.com
Contact: +92-3339116086

osteoclasts and inhibition of osteoblastic activity. It is believed that hepatocyte growth factor (HGF) secreted by multiple myeloma cells inhibits BMP (bone morphogenetic protein) and suppresses the transcription factor RUNX2, necessary for the formation and differentiation of osteoblasts from mesenchymal stem cells.[4] Apart from the secretions of growth factors, the attachment of multiple myeloma cells to the cells of the BMM triggers a variety of proliferative and antiapoptotic signaling pathways, which leads to multiple myeloma growth and survival. Hepatocyte growth factor(HGF)/Scatter factor(S.F.) is a multifunctional cytokine that promotes cell proliferation, survival, motility, scattering, differentiation, and morphogenesis.[5] Subsequently, it was found that S.F. was similar to HGF.[6] Further, an experimental study established HGF/SF as the ligand for MET receptor tyrosine kinase[7] encoded by a proto-oncogene c-MET.[8]

It was revealed that activation of this Oncogene involved a chromosomal translocation that linked a segment known as the translocated promoter region (TPR) on chromosome1 to the c-terminal of the MET gene on chromosome 7. The outcome of this translocation was a truncated receptor (TPR-MET) having constitutively active tyrosine kinase activity.[9] The receptor c-Met is expressed in epithelial cells, and its ligand HGF is produced by surrounding mesenchymal cells. In this way, both of them play an important role in mediating signals given by mesenchymal cells and received by epithelial cells.[10,11]

One of the final steps of metastasis is the transendothelial migration of cancer cells. HGF is involved in this process by enhancing the expression of adhesion molecules both on cancer cells and endothelial cells. HGF induces endothelial expression of CD44 cell surface glycoproteins, involved in Cell to cell interactions, adhesion, and migration.[12] Also, the up-regulation of CD44 by HGF has been reported in breast cancer cells.[13] Similarly, HGF also promotes cell adhesions by increasing the affinity of integrins for their specific ligands[14] or enhancing their expression.[15]

RNA interference is an evolutionarily conserved process that cells use to turn down or silence the activity of specific genes at transcriptional and post-transcriptional levels. In functional genomics, RNAi has emerged as a widespread and essential research tool for interrogating the functions of genes and probing their roles in complex biological pathways. This technique can reveal key participants of normal and disease processes and may lead scientists to new drug targets.[16] RNAi can be exploited in experimental settings by the exogenous introduction of dsRNAs or constructs which express shRNAs (short hairpin RNA). The shRNAs produce sustained gene suppression and can transfect difficult cell lines, e.g., primary cells and nondividing cells such as neurons.[17,18]

This work aimed to clone shRNA oligos into a retroviral vector, using HGF as a model system. Secondly, the aim was to analyze myeloma cell lines stably transduced with HGF-shRNA.

MATERIALS AND METHODS

The 97-mer oligos (eight in number) against the HGF mRNA sequence (Accession number: NM_000601) were designed using web-based software.[19] Samples were either used directly in PCR or frozen at -20 °C for later use. A list of oligos targeting HGF mRNA is given in Table 1. The 97 mer-oligos were amplified using the universal primers of a 5'-miR30 PCR XhoI as a forward primer and 3'-miR30 PCR EcoRI as the reverse primer. These primers add XhoI and EcoRI sites in an amplified product, crucial for cloning into miR30 based MSCVP_2GmFF vector. The cloning of amplified oligos was carried out using standard cloning protocol. The cloned fragments were sequenced for their proper orientation and sequenced using the Big Dye terminator cycle sequencing kit (Applied Biosystems, Foster City, C.A.) according to the manufacturer's protocol.

INA-6 control, INA-6-HGF-1, and INA-6-HGF-2 were maintained by feeding them twice a week with their specific growth media as described. This was done by directly diluting the cells in the culture flasks and continue expanding them or by removing a portion of cells from culture flasks and diluting the remaining cells down to a seeding density of 200000 cells/ml.

For the HGF knockdown experiment, the human myeloma cell line JJN-3 was selected. Seven different cell lines were established in the laboratory by stable transduction of JJN-3 cells with lentiviral transduction particles (MISSION®, Sigma Aldrich). Five of them were produced due to infection with

HGF-shRNA lentiviral particles, while the other two cell lines were used as controls. Briefly, shRNA lentiviral particles were transduction-ready viral particles used for gene knockdown in mammalian cells, both in dividing and nondividing cells. shRNAs targeting a specific gene were sequence-verified and then cloned into a lentiviral vector pLKO.1-puro. The MISSION® control transduction viral particles containing non-targeting shRNA and not having a knockdown effect on any mammalian gene were used to negative control cell line. The Turbo GFP particles expressing turbo GFP under the CMV promoter were used as a positive control of transduction.

RNA was isolated from different cell lines. To measure the HGF mRNA expression, qRT-PCR was run on step one plus a Real-time PCR system with TaqMan® universal PCR master mix and TaqMan®-gene expression assay (HGF and GAPDH, Applied biosystems). GAPDH was used as an internal reference gene. The data was analyzed by Applied biosystem step one plus software, which used the standard comparative ΔΔCT method to determine relative changes in HGF mRNA.

To visualize the HGF proteins, western blot analysis was done using different cell lines. Around 50 ml of cell lines were centrifuged at 1500 rpm for 8 minutes at room temperature. The pelleted cells were resuspended in 40 µl lysis buffer and kept on ice for 20-30 min. The lysis buffer used, contained 1% Nonidet-P40 (NP-40)(Sigma Aldrich), 150 mM NaCl, 50 mM Tris-HCL (pH 7.5)(complete mini-tablet)(Roche), 1mM Na_3VO_4 and 50 mM NaF.

The protein concentration was measured using the Micro BCA Protein Assay Kit (Thermo scientific). An amount of 20.2 µg of protein was denatured by heating for 10 min at 70 °C in the presence of 4 X NuPAGE LDS sample buffer (Novex by life technologies) and 100 mM DTT and 6 µl of See Blue plus two pre-stained standards were separated on 10 %Bis-Tris-NuPAGE gel (life technologies) using 1X MOPS Running buffer (life technologies).

Cell line cultures with around 150000 cells/ml were collected on day two and day 3. Cell cultures were centrifuged @ 1500 rpm for 8 min at room temperature. About 1 ml supernatant of each cell culture was collected in a 1.5 ml Eppendorf tube, and samples were frozen at -20 °C till later use.

Capture antibody (anti-HGF) was diluted in PBS to a working concentration of 1 µg/ml. A 96 well microplate (COSTAR EIA plate) was coated with 50 µl/well of diluted capture antibody. A volume of 50 µl of recommended working solution of streptavidin-HRP diluted in reagent diluent to 200 ng/ml was used. The optical density of each well was determined using a microplate reader at 450 nm. The concentration was estimated from the standard curve using linear regression.

RESULTS

For the establishment of retroviral HGF-shRNA expression constructs, eight different 97 base pairs shRNA oligos were designed against human HGF mRNA (NM_000601) using the web-based software19 (Table 1). These oligos were amplified by polymerase chain reaction using forward and reverse primers containing XhoI and EcoRI cloning sites (Figure 1). This allowed cloning into XhoI and EcoRI cloning sites of the retroviral vector MGCVP2GmFF.

Two to four clones from each LA-plate (excluding the control plates) were grown in L.B. culture media containing ampicillin and purified using a plasmid mini kit. To check the cloning of the desired inserts, HGF-shRNA transformants were digested with NotI and PmeI. The entire miR30-shRNA construct was released using these enzymes. The NotI restriction enzymes cut at 2855 base pairs, while PmeI cut the vector MSCVP2GmFF at 3245 base pairs[20], thus generating two DNA bands with a size of 390 bp and 7.2 kb (Figure 2). As shown, clones a, c, e, f, g, i, k, l, o, and p produced the expected bands with NotI and PmeI restriction (~ 400 bp and ~7-8 kb) (Figure 2). The transformants with the correct sizes of released fragments using NotI and PmeI were sequenced using EGFP-C universal primer. The sequence of the HGF-shRNA oligo in the transformants was compared to the sequence of designed/desired oligo HGF-shRNA sequences (Table 2). Nucleotide sequence alignment showed that HGF-shRNA oligo-1401, HGF-shRNA oligo-374, HGF-shRNA oligo-882, HGF-shRNA oligo-1292, and HGF-shRNA oligo-1137 were ligated within MSCVP2GmFF. Thus a total of five different HGF-shRNA expression constructs were generated.

JJN-3 is a myeloma cell line producing a large amount of HGF protein. JJN-3 cells transduced with lentiviral particles containing shRNA oligos towards

the HGF gene were developed in this study. Seven different cell lines were made, five having different shRNAs towards the HGF gene, a negative control (JJN-3-sh nontarget) cell line, and positive control (JJN-3-sh turbo GFP) of the transduction cell line (Table 2). To examine the possible effects of different shRNAs (carried in lentiviral transduction particles) on HGF mRNA levels, HGF mRNA expression was analyzed by quantitative RT-PCR. Both the transduced cell lines JJN-3-sh3308 and JJN-3-sh47137 showed an almost 60% reduction in HGF mRNA level relative to that of the negative control (JJN-3-sh Turbo GFP). While the cell lines JJN-3-sh 3308, JJN-3-sh3307and JJN-3-sh3309 did not significantly reduce HGF mRNA expression (Figure 3).

After the analysis of HGF mRNA expression, Western blot was performed to examine HGF protein level expression. HGF protein shows typical three bands ~90 kDa band of pro-HGF, ~60 kDa band of α HGF chain, and ~30 kDa band of β HGF chain.[21] To visualize HGF protein bands, JJN-3-sh nontarget, and JJN-3-Turbo GFP and ANBL-6 were used as positive controls for the presence of typical HGF protein bands. At the same time, the non-HGF producing cell line INA-6-control was used as a negative control. The lentivirally transduced cell lines JJN-3-sh3308 and JJN-3-sh47137 gave typical bands of HGF proteins; however, their intensity was low compared to those found in JJN-3-sh nontarget and JJN-3-sh Turbo GFP (Figure 4). These findings agree with the knockdown effects of the respective shRNAs on hepatocyte growth factor at the mRNA level. However, the knockdown effect was not 100% compared to the negative control cell line (INA-6-control), as shown in Figure 4.

To calculate the amount of secreted HGF protein by JJN-3sh3308, JJN-3-sh4713, an equal number of cells (150000 cells/ml) from the cell lines JJN-3, JJN-3-sh3308, JJN-3-sh nontarget, ANBL-6, and U266 were cultured in their specific cell culture media. Cell counts/ml of culture media was calculated for each cell line at day two and day 3. The cell lines JJN-3 and JJN-3-sh nontarget were used as positive controls for HGF secretion, while the cell lines INA-6 and INA-6 control were negative controls (they do not secrete HGF). Two other myeloma cell lines were also included; U266, which produces only a small amount of HGF, and ANBL-6, which provide a substantial HGF. The concentration of HGF protein secreted by JJN-3-sh3308 and JJN-3-sh47137 was lower than the control cell lines JJN-3 and JJN-3-sh nontarget, indicating a knockdown effect (Figure 5). On day two, there was ~70% lower secretion of HGF in the two knockdown cell lines than in control, while on day three, it was ~50% lower (Figure 5). As expected, very low HGF concentration was detected in cell supernatants of U266. Taking the cell number into account (data not are shown), the HGF knockdown cells secreted approximately the same amount of HGF as the ANBL-6 cells.

DISCUSSION

RNAi is generally used in biomedical research to study the outcome of a blocked/knockout gene. Over the past decades, significant advances have been made in RNAi methods. Huge data on genes has been collected using this technology, and in some cases, important observations have been made about genes dysregulated in different pathologies. The mechanism of RNAi is built on sequence-specific degradation of host mRNA through the delivery of a double-stranded RNA, identical to the target sequence.[22,23] The introduction of shRNA into mammalian cells through either transfection or infection with viral vectors allows stable integration of shRNA and long-term down-regulation of the targeted gene.

This study aimed to establish a cloning method for RNAi in the myeloma group. HGF is an important protein in multiple myeloma. Therefore HGF was chosen as a model system to establish the shRNA cloning method. We wanted to generate shRNAs towards the HGF gene that could be used via retroviral transduction of myeloma cells. Growth and purification of clones, following sequencing, and NCBI nucleotide blast alignment showed that five out of eight HGF-shRNAs had been ligated into the vector (Figure 2). The cloning procedure took more than expected time. Therefore as an alternative in parallel, five different lentiviral particles containing shRNA towards HGF genes (Table 2), premade by the company Sigma Aldrich, were ordered. The HGF-shRNA lentiviral particles were transduced into the high HGF producing myeloma cell line JJN-3, together with control particles. In this work, JJN-3 cell lines stably transduced with shRNAs against the HGF gene were tested for the downregulation of HGF protein (Figure 4). The knockdown effect was analyzed both at the mRNA level, protein lev-

Table 1: List of Oligos targeting HGFmRNA

HGF-shRNA Oligos	Sequence (5`-3`)
HGF-Oligo944	TGCTGTTGACAGTGAGCGAACTGTCAATACCATTTGGAATTAGTGAAGCCACAGATGTA-ATTCCAAATGGTATTGACAGTGTGCCTACTGCCTCGGA
HGF-Oligo1401	TGCTGTTGACAGTGAGCGAGTTGTGAAGGTGATACCACACTAGTGAAGCCACAGATG-TAGTGTGGTATCACCTTCACAACGTGCCTACTGCCTCGGA
HGF-Oligo374	TGCTGTTGACAGTGAGCGCAGAAACTGCATCATTGGTAAATAGTGAAGCCACAGATG-TATTTACCAATGATGCAGTTTCTATGCCTACTGCCTCGGA
HGF-Oligo882	TGCTGTTGACAGTGAGCGAACACTGATGTTCCTTTGGAAATAGTGAAGCCACAGATG-TATTTCCAAAGGAACATCAGTGTCTGCCTACTGCCTCGGA
HGF-Oligo1292	TGCTGTTGACAGTGAGCGCGCAAGTAAGCTGAATGAGAATTAGTGAAGCCACAGATGTA-ATTCTCATTCAGCTTACTTGCATGCCTACTGCCTCGGA
HGF-Oligo1137	TGCTGTTGACAGTGAGCGCTTCCAAACTGTGATATGTCACTAGTGAAGCCACAGATG-TAGTGACATATCACAGTTTGGAATTGCCTACTGCCTCGGA
HGF-Oligo1389	TGCTGTTGACAGTGAGCGCGCCCTATTTCTCGTTGTGAAGTAGTGAAGCCACAGATG-TACTTCACAACGAGAAATAGGGCATGCCTACTGCCTCGGA
HGF-Oligo1937	TGCTGTTGACAGTGAGCGCCGAGGGAAGGTGACTCTGAATTAGTGAAGCCACAGATGTA-ATTCAGAGTCACCTTCCCTCGATGCCTACTGCCTCGGA

* Sequences in red represent the flanking miR-30 sequences common to each oligo. Sequences in green represent the miR-30 loop sequence. Sequences in pink and black color represent the sense, and antisense (respectively) selected target sequences of shRNA against the HGF gene.

Table 2: List of shRNA transduction ready lentiviral particles

Lentiviral transduction Particles	The sequence of sh-RNA targeting the sequence of Human Hepatocyte growth factor gene in lentiviral transduction particles	Transduce cell line
TRCN0000003306	CCGGCCCGTAATATCTTGTGCCAAACTCGAGTTTGGCACAAGATATTACGG-GTTTTT	JJN-3-sh 3306
TRCN0000003307	CCGGCAGACCAATGTGCTAATAGATCTCGAGATCTATTAGCACATTGGTCT-GTTTTT	JJN-3-sh 3307
TRCN0000003308	CCGGGCAAAGACTACCCTAATCAAACTCGAGTTTGATTAGGG-TAGTCTTTGCTTTTT	JJN-3-sh3308
TRCN0000003309	CCGGGCAAGTAAGCTGAATGAGAATCTCGAGATTCTCATTCAGCT-TACTTGCTTTTT	JJN3-3-sh3309
TRCN0000047137	CCGGGCAAAGACTACCCTAATCAAACTCGAGTTTGATTAGGG-TAGTCTTTGCTTTTTG	JJN-3-sh47137
Nontargeting sh RNA–negative control of knockdown	contains a sh-RNA sequence that only activates RISC and RNAi pathway without targeting any mammalian gene. Acts as a negative control of gene knockdown.	JJN3-sh nontarget
Positive control of transduction	Contains no shRNA sequence, positive control of transduction with reporter gene turbo GFP	JJN3-turboGFP

* Having specific shRNA sequences against human Hepatocyte growth factor gene) along with controls. TRC (RNAi consortium) represents the Library number of lentiviral shRNA particles.

el, and by looking at the amount of secreted HGF (Figure 3-5). Two out of five shRNAs (HGF- shRNA transduction viral particles (TRCN0000003308 and TRCN0000047137) gave 50 to 60% knockdown in HGF mRNA (Figure 3). The knockdown effect in these cell lines (termed JJN-3-sh3308 and JJN-3-sh47137) was also verified by Western Blot analysis (Figure 4). We got typical bands of HGF protein (pro-HGF, α chain, and β chain), but it was agreed that the intensity of these bands was not that high as those of the positive control cell lines (JJN-3 Turbo GFP and JJN-3-sh nontarget) as cab observed in Figure 4.

We also managed to verify the knockdown effect by measuring secreted HGF via ELISA (Figure 5). There was approximately 70% reduction at day 2

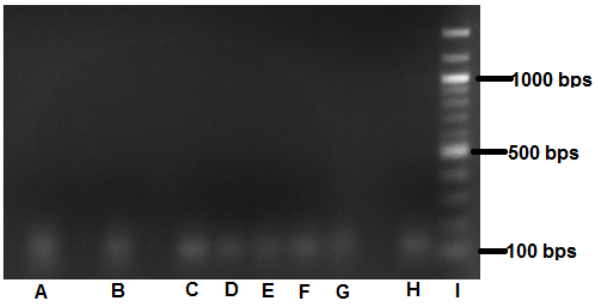

Figure 1: Gel picture of HGF-shRNA oligos.A: HGF-shRNA oligo-944, B: HGF-shRNA oligo-1404, C: HGF-shRNA oligo-374, D: HGF-shRNA oligo-882, E: HGF-shRNA oligo-1292, F: HGF-shRNA oligo-1137, G: HGF-shRNA oligo-1389, H: HGF-shRNA oligo-1937, I: 100 base pairs DNA ladder, 1.8% agarose gel. [DNA = 5 µl (4 ng/µl)].

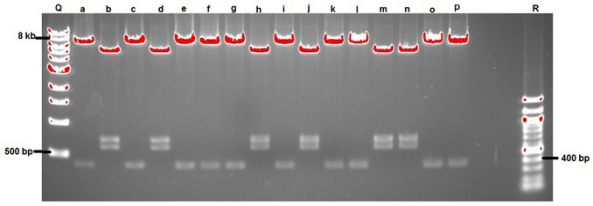

Figure 2: NotI and PmeI restriction cut of HGF-shRNA transformation clones. Transformation clones a, b: HGF-shRNA oligo-994, c, d: HGF-shRNA oligo-1401, e, f: HGF-shRNA oligo-374, g, h: HGF-shRNA oligo-882, i, j: HGF-shRNA oligo-1292, k, l: HGF-shRNA oligo-1137, m, n: HGF-shRNA oligo-1389, o, p: HGF-shRNA oligo-1937, Q: 1kb DNA ladder, R: 100 bp DNA ladder. 1.2% agarose gel was used.

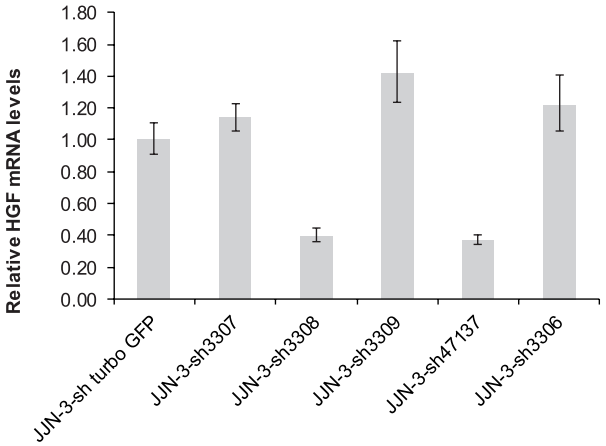

Figure 3: The expression level of HGF mRNA in the HGF-shRNA transduced cells were determined by qRT-PCR. Fold changes in HGF mRNA was calculated relative to JJN-3-Turbo GFP using the ΔΔ Ct method. One representative experiment out of three, with three replicas (±SD), is shown.

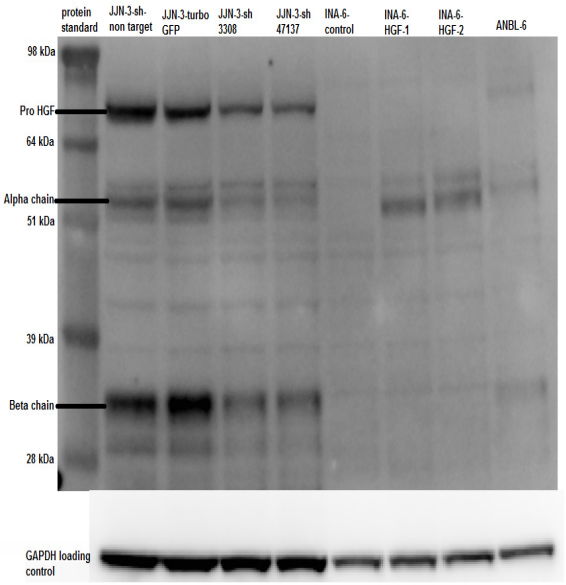

Figure 4: Western Blot analysis of HGF down-regulation and HGF up-regulation. 90 kDa pro-HGF, 60 kDa α HGF chain, 30 kDa β HGF chain.

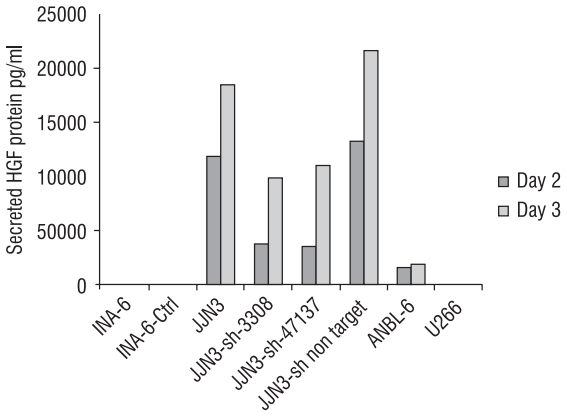

Figure 5: HGF secretion by different myeloma cell lines. INA-6 and INA-6-control were negative controls while JJN-3, JJN-3-sh nontarget, and ANBL-6 were positive controls for HGF secretion. The graph represents a secreted HGF protein present in 1 ml of cell supernatant. One biological experiment with two replicas (±SD) is shown.

and 50 % at day 3 in the secretion of HGF protein by these knockdown cell lines compared to control cell lines JJN-3 and JJN-3-sh nontarget. Although we managed to get a knockdown effect on the HGF level, the concentrations of HGF protein secreted by JJN-3 cells were still very high. This was observed when we compared the amount of secreted HGF from these cell lines with other HGF producing cells, such as the ANBL-6 cells and the INA-6 HGF overexpressing

cells (data not shown). The possible reason for this finding could be that many available target transcripts may be more resistant to RNAi mediated silencing.[25] To obtain further knockdown, it could be possible to combine several different shRNA constructs that have a significant degradation of HGF mRNA when used alone.

CONCLUSION

Five different retroviral-HGF-shRNA expression constructs have been made. These constructs will hopefully contribute to the understanding of HGF response via gene knockdown studies in myeloma cell lines. We were able to establish JJN-3 cells with HGF knockdown. Although a good knockdown effect was observed, there are still high amounts of HGF produced in these cells, so the biological effects of this knockdown remain to be shown. The HGF knockdown transduced cell lines (JJN-3-sh3308 and JJN-3-sh47137) can be further studied to obtain more insight into the pathology of multiple myeloma HGF.

REFERENCES

1. R.A. Kyle, Multiple myeloma: an odyssey of discovery, British Journal of Haematology, 111 (2000) 1035-1044.
2. R. Siegel, E. Ward, O. Brawley, A. Jemal, Cancer statistics, 2011: the impact of eliminating socioeconomic and racial disparities on premature cancer deaths, CA: a cancer journal for clinicians, 61 (2011) 212-236.
3. N. Becker, Epidemiology of multiple myeloma, Recent results in cancer research. Fortschritte der Krebsforschung. Progres dans les recherches sur le cancer, 183 (2011) 25-35.
4. T. Standal, N. Abildgaard, U.M. Fagerli, B. Stordal, O. Hjertner, M. Borset, A. Sundan, HGF inhibits BMP-induced osteoblastogenesis: possible implications for the bone disease of multiple myeloma, Blood, 109 (2007) 3024-3030.
5. C. Birchmeier, W. Birchmeier, E. Gherardi, G.F. Vande Woude, Met, metastasis, motility and more, Nat Rev Mol Cell Biol, 4 (2003) 915-925.
6. K.M. Weidner, N. Arakaki, G. Hartmann, J. Vandekerckhove, S. Weingart, H. Rieder, C. Fonatsch, H. Tsubouchi, T. Hishida, Y. Daikuhara, et al., Evidence for the identity of human scatter factor and human hepatocyte growth factor, Proc Natl Acad Sci U S A, 88 (1991) 7001-7005.
7. D.P. Bottaro, J.S. Rubin, D.L. Faletto, A.M. Chan, T.E. Kmiecik, G.F. Vande Woude, S.A. Aaronson, Identification of the hepatocyte growth factor receptor as the c-met proto-oncogene product, Science, 251 (1991) 802-804.
8. L. Naldini, E. Vigna, R.P. Narsimhan, G. Gaudino, R. Zarnegar, G.K. Michalopoulos, P.M. Comoglio, Hepatocyte growth factor (HGF) stimulates the tyrosine kinase activity of the receptor encoded by the proto-oncogene c-MET, Oncogene, 6 (1991) 501-504.
9. M. Park, M. Dean, C.S. Cooper, M. Schmidt, S.J. O'Brien, D.G. Blair, G.F. Vande Woude, Mechanism of met oncogene activation, Cell, 45 (1986) 895-904.
10. E. Sonnenberg, D. Meyer, K.M. Weidner, C. Birchmeier, Scatter factor/hepatocyte growth factor and its receptor, the c-met tyrosine kinase, can mediate a signal exchange between mesenchyme and epithelia during mouse development, Journal of Cell Biology, 123 (1993) 223-235.
11. M. Stoker, E. Gherardi, M. Perryman, J. Gray, Scatter factor is a fibroblast-derived modulator of epithelial cell mobility, Nature, 327 (1987) 239-242.
12. S. Hiscox, W.G. Jiang, Regulation of Endothelial CD44 Expression and Endothelium–Tumour Cell Interactions by Hepatocyte Growth Factor/Scatter Factor, Biochemical and Biophysical Research Communications, 233 (1997) 1-5.
13. S. Mine, T. Fujisaki, C. Kawahara, T. Tabata, T. Iida, M. Yasuda, T. Yoneda, Y. Tanaka, Hepatocyte growth factor enhances adhesion of breast cancer cells to endothelial cells in vitro through the up-regulation of CD44, Experimental Cell Research, 288 (2003) 189-197.
14. L. Trusolino, S. Cavassa, P. Angelini, M. Ando, A. Bertotti, P. Comoglio, C. Boccaccio, HGF/scatter factor selectively promotes cell invasion by increasing integrin avidity, The FASEB Journal, 14 (2000) 1629-1640.
15. N. Kawakami-Kimura, T. Narita, K. Ohmori, T. Yoneda, K. Matsumoto, T. Nakamura, R. Kannagi, Involvement of hepatocyte growth factor in increased integrin expression on HepG2 cells triggered by adhesion to endothelial cells, British journal of cancer, 75 (1997) 47-53.
16. G.J. Hannon, RNA interference, Nature, 418 (2002) 244-251.
17. G.D. Fewell, K. Schmitt, Vector-based RNAi approaches for stable, inducible and genome-wide screens, Drug Discovery Today, 11 (2006) 975-982.
18. J. Robert E. Farrell, RNA methodologies. A laboratory guide for RNA isolation and characterization, 4th ed., Academic Press, London, 2010.
19. shRNA Design, Accessed on March 02, 2013, http://cancan.cshl.edu/RNAicentral/RNAi.cgi?type=shRNA.
20. MSCV P2Gm FF: Sequences, Accessed on March 17, 2013, http://www.addgene.org/19750/sequences/.
21. R. van der Voort, T. E.I. Taher, P.W.B. Derksen, M. Spaargaren, R. van der Neut, S.T. Pals, The hepatocyte growth factor/ met pathway in development, tumorigenesis, and B-cell differentiation, in Advances in Cancer Research, Academic Press, 2000, pp. 39-90.

22. C.B. Moore, E.H. Guthrie, M.T. Huang, D.J. Taxman, Short hairpin RNA (shRNA): design, delivery, and assessment of gene knockdown, Methods Mol Biol, 629 (2010) 141-158.

23. G.D. Fewell, K. Schmitt, Vector-based RNAi approaches for stable, inducible, and genome-wide screens, Drug Discov Today, 11 (2006) 975-982.

24. Hairpin Cloning, Accessed on March 18, 2013, http://web.mit.edu/hyneslab/flipvectors/.

25. E. Larsson, C. A.U. - Sander, D. A.U. - Marks, mRNA turnover rate limits siRNA and microRNA efficacy, Mol Syst Biol, 6 (2010).