DEVELOPMENT OF MULTIPLE MYELOMA CELL LINES WITH HIGH EXPRESSION OF HEPATOCYTE GROWTH FACTOR

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ABSTRACT

Objective: *To determine the overexpress HGF gene in non HGF producing humane myeloma cell lines (INA-6).*

Materials and Methods: *In this study, retroviral- expression constructs were designed and constructed. The sequence of the cloned fragments in the vector constructs was determined and the effect of construct was examined using qRT-PCR and Western blot analysis. At department of cancer research & molecular medicine, norwegien university of science & technology, Trondheim Norway, in June 2016 to April 2018.*

Results: *We established expression constructs based on HGF mRNA sequence. The HGF non producing human myeloma cell line INA-6 transduced with lentiviral HGF-overexpression transduction particles, were analyzed for their effect on the HGF gene expression. HGF overexpression was analyzed both at mRNA level and protein levels. Two myeloma cell line INA-6 HGF-1 and INA-6HGF-2 showed significant over expression of HGF mRNA and protein.*

Conclusion: *In conclusion, the HGF overexpression cell lines can be used as tissue culture models to investigate the role of HGF protein in pathology of multiple myeloma.*

Keywords: *Multiple myeloma, Hepatocyte growth factor (HGF), Gene, Overexpression*

INTRODUCTION

Multiple myeloma is a devastating disease which gained curiosity of physicians and scientists for decades.[1] The well-known early observations about the disease were made in mid-19th century in the case of 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 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 over activity of 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 which is necessary for formation and differentiation of osteoblasts from mesenchymal stem cells.[4] Apart from secretions of growth factors, the attachment of multiple myeloma cells to the cells of the BMM, trigger a variety of proliferative and antiapoptotic signaling pathways which leads to multiple myeloma growth and survival. Hepatocyte growth factor(HGF)/Scatter factor(SF), is a multifunctional cytokine involved in cell proliferation, survival, motility, scattering,

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differentiation and morphogenesis.[5] Experimental studies established that HGF/SF is the ligand for MET receptor tyrosine kinase[6] encoded by a proto oncogene c-MET.[7] It was revealed that activation of this oncogene involved a chromosomal translocation resulting in a truncated receptor (TPR-MET) with active tyrosine kinase activity.[8] The receptor c-Met is expressed in epithelial cells and its ligand HGF is produced by surrounding mesenchymal cells interconnecting mesenchymal and epithelial cell signalling.[9,10]

One of the final steps of metastasis is the trans endothelial 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.[11] Also up regulation of CD44 by HGF has been reported in breast cancer cells.[12] Similarly HGF also promotes cell adhesions by increasing affinity of integrins for their specific ligands[13] or enhancing their expression.[14]

The aim of this work was to clone HGF into a retroviral vector and to analyze HGF non producing myeloma cell lines stably transduced with HGFover-expression vector.

MATERIALS AND METHODS

INA-6 cells transduced with HGF expression plasmids previously made in laboratory using HGF mRNA sequence (Accession number:NM_000601.4) The cloning of amplified HGF coding region was carried out using standard cloning protocol. The cloned fragment was sequenced for their proper orientation and sequence using Big Dye terminator cycle sequencing kit (Applied biosystems, Foster city, CA) according to 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.

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

In order to visualize the HGF proteins, western blot analysis was done using different cell lines. Around 50 ml of a cell lines was centrifuged 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 Na3VO4 and 50 mM NaF.

The protein concentration was measured using 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 2 pre stained standard 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 at day 2 and day 3. Cell cultures were centrifuged @ 1500 rpm for 8 min at room temperature. Around 1 ml supernatant of each cell culture was collected in 1.5 ml eppendrof tube and samples were frozen at -20 °C till later use.Capture antibody (anti-HGF) was diluted in PBS to working concentration of 1 µg/ml. A 96 well micro plate (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 for each well. The optical density of each well was determined using micro plate reader at 450 nm. The concentration was estimated from the standard curve using linear regression.

RESULTS

HGF expression in INA-6-HGF-1 and INA-6-HGF-2 cell lines

INA-6 is a myeloma cell line that does not produce HGF. INA-6 cells transduced with HGF expression plasmids were previously made in our laboratory, using retroviral transduction. These expression plasmids contained a full length HGF cDNA sequence (HGF transcription variant 1: Acc. nr: NM_000601.4). Two different cell clones (INA-6-HGF-1 and INA-6-HGF-

2) Had been picked and grown (after dilution cloning). In addition one control clone (INA-6 control), that was made by retroviral transduction of empty plasmid (no HGF gene) was also grown. In order to examine the expression of HGF mRNA in the non HGF producing INA-6 cell line transduced with HGF expression vector, RNA was isolated from the cell lines INA- 6, INA-6-control, INA-6-HGF-1, INA-6-HGF-2, JJN-3 and ANBL-6. RNA was reversely transduced to cDNA and total HGF mRNA expression was analyzed by qRT- PCR. The cell lines INA-6 and INA-6-control were used as negative controls while the positive controls were the HGF producing myeloma cell lines JJN-3 and ANBL-6. Results are shown in Figure

1. The result shows high levels of HGF mRNA in transduced cell lines INA-6-HGF-1 and INA-6-HGF-2 and no HGF in the INA-6 control cell line. The HGF mRNA levels in INA-6- HGF-1 and INA-6-HGF-2 were higher than in the high HGF producing cell line JJN-3.

HGF protein analysis in INA-6 cells

After 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. In order to visualize HGF protein bands, JJN-3-sh non target and JJN-3-Turbo GFP and ANBL-6 were used as positive controls for the presence of typical HGF protein bands. While the non HGF producing cell line INA-6-control was used as negative control. The cell lines INA-6-HGF-1 and INA-6-HGF-2 had a strong intensity band approximately at the size of the HGF α-chain (Figure 2). The INA-6 control cell lines, had no HGF bands as expected. It was strange to notice that HGF overexpressing cells did not have any band with the size of β- chain or the pro HGF. The α-chain band in the HGF overexpressing cell lines observed to be a bit smaller than the α-chain bands in the JJN-3 and ANBL-6 cell lines (Figure 2).

Secretion of HGF protein validated by ELISA

The over expression of HGF protein in INA-6-HGF-1 and INA-6-HGF-2 was confirmed both at the mRNA and at the protein level. This was a motivating fact to further investigate the secretion of HGF protein by these cell lines. This goal was achieved by ELISA experiment. In order to calculate the amount of secreted HGF protein by INA-6-HGF-1 and INA-6-HGF-2, equal number of cells (150000 cells/ml) from the cell lines (INA-6, INA- 6-control, INA-6-HGF-1, INA-6-HGF-2, JJN-3, ANBL-6 and U266), were seeded in their specific cell culture media. Cell counts/ml of culture media was calculated for each cell line followed by collection of 1 ml of cell supernatant at day 2 and day 3. Supernatants were analyzed by ELISA for the presence of HGF protein. The cell lines JJN-3 and JJN-3-sh non target were used as positive controls for HGF secretion, while the cell lines INA-6 and INA-6 control were negative controls as these do not secrete HGF. In addition two other myeloma cell lines were included, U266 which produce only small amount of HGF and ANBL-6 which produce substantial amount of HGF, although not as high amount as JJN-3. Total concentration of secreted HGF protein, along with total number of cells (of each cell line) is listed in Table 1. HGF protein was found in the supernatants of HGF over expressing cell lines INA-6-HGF-1 and INA-6-HGF-2 as compared to the negative controls INA-6 and INA-6-control. However, the concentrations were lower than the HGF producing cell lines ANBL-6 and JJN-3. As expected, very low HGF concentration was detected in cell supernatants of U266.

Result of growth curve experiment

The over expression of HGF mRNA and secretion of HGF protein by INA-6-HGF-1 and INA-6-HGF-2 were encouraging facts to rule out if they had any growth advantage over the non HGF producing myeloma cell lines. For this purpose their growth was monitored for 11 days by counting cells/ml every 24 h. INA-6-control and INA-6 were used as controls. Results are shown in Figure 4. It was found that the cell lines INA-6 was growing faster than the transduced cell lines INA-6-HGF-1, INA-6-HGF-2 and INA-6-control. The transduced cell lines were following almost similar growth curves. Thus, overexpression of HGF does not seem to give the cells

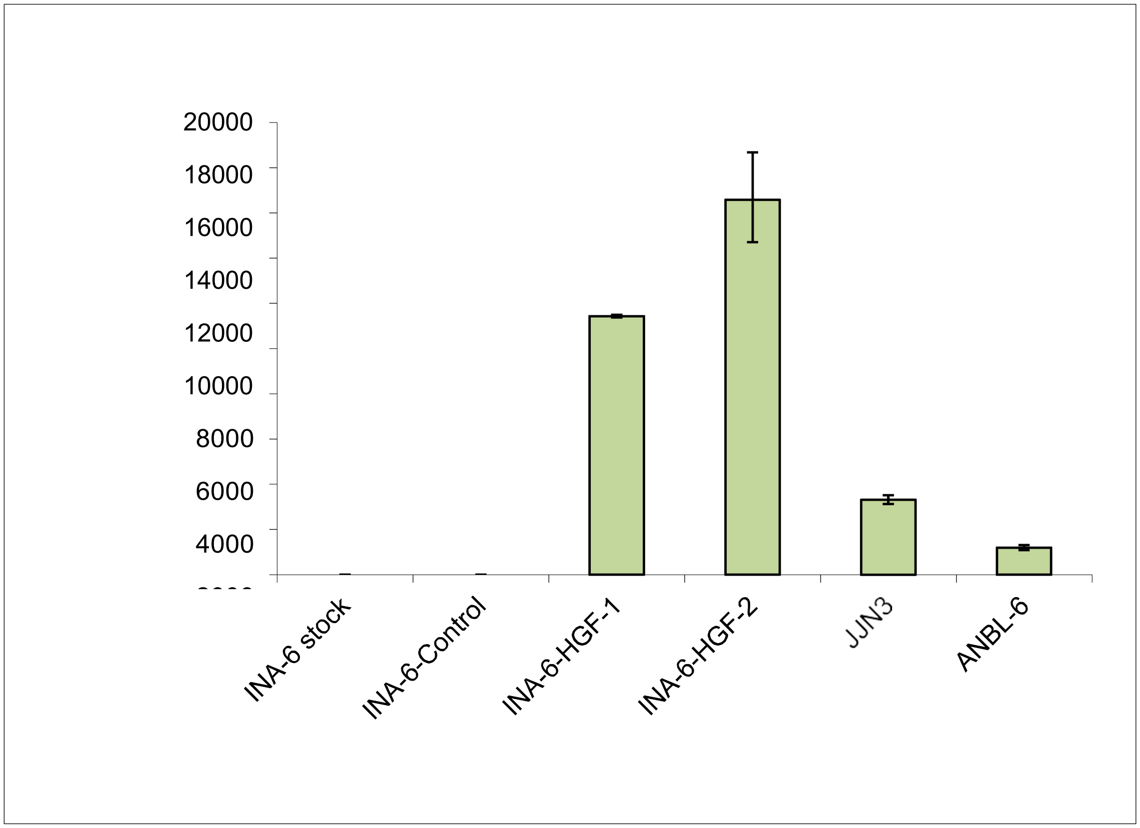

Fig 1: Expression levels of HGF mRNA in the transduced cell line INA-6-HGF-1 and INA-6-HGF-2. HGF producing cell lines JJN-3 and ANBL-6 were included as positive controls. INA-6-control and untransduced INA-6-cells were used as negative controls. Fold induction in mRNA was analyzed by qRT-PCR using ΔΔCt method. One biological experiment with 3 replica (±SD). One representative experiment out of three is shown.

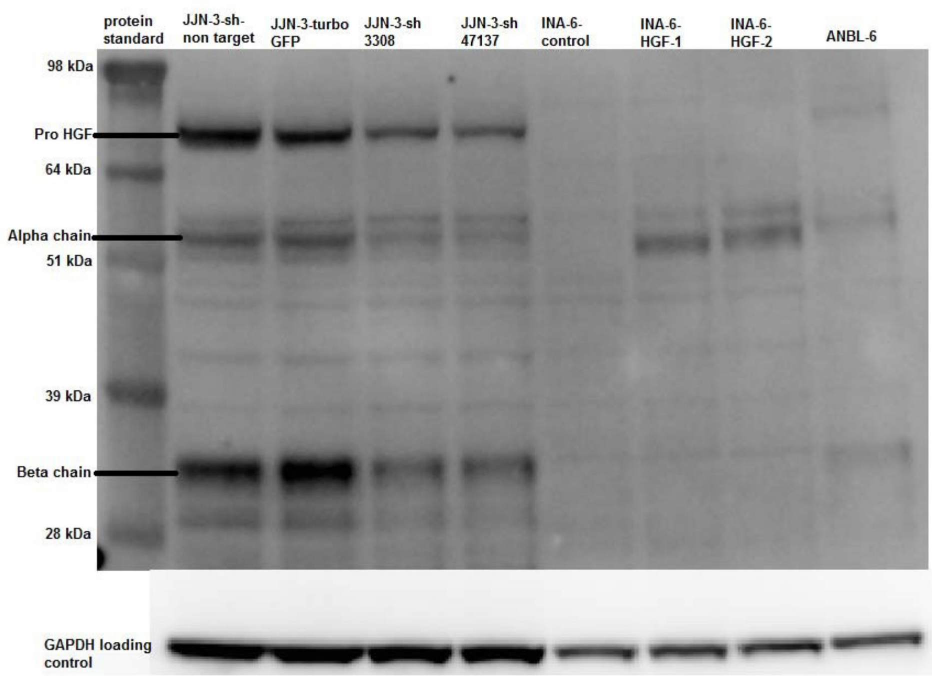

Fig 2: Western Blot anlaysis of HGF down regulation and HGF up regulation. 90 kDa pro HGF, 60 kDa α HGF chain, 30 kDa β HGF chain.

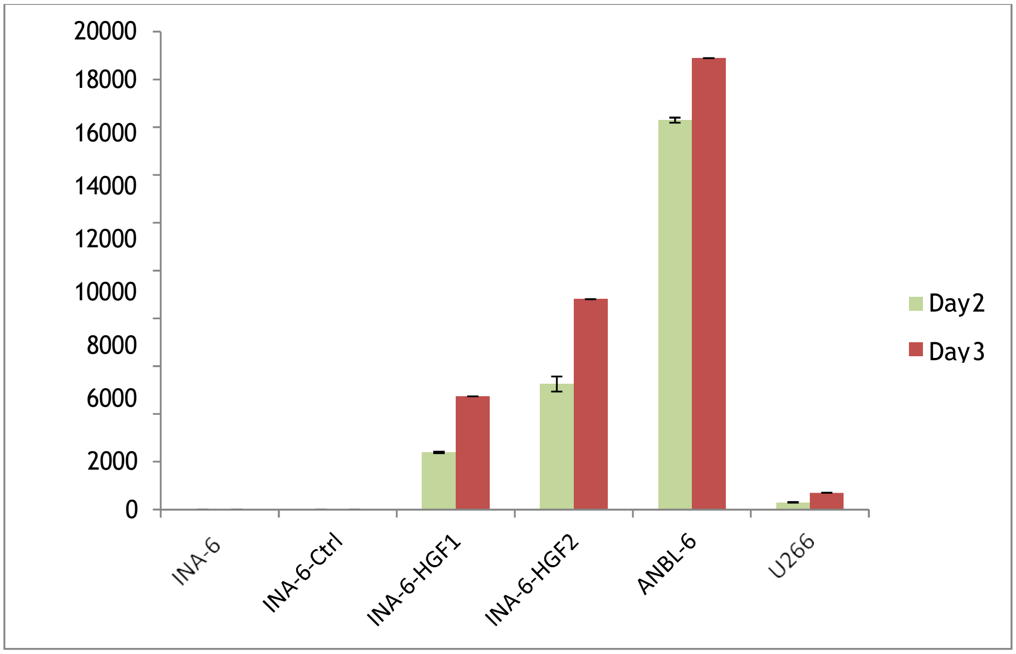

Fig 3: HGF secretion by different myeloma cell lines. INA-6 and INA-6-control were negative controls while ANBL-6 was positive control for HGF secretion.The graph represents secreted HGF protein present in 1 ml of cell supernatant. One biological experiment with two replica (±SD) is shown.

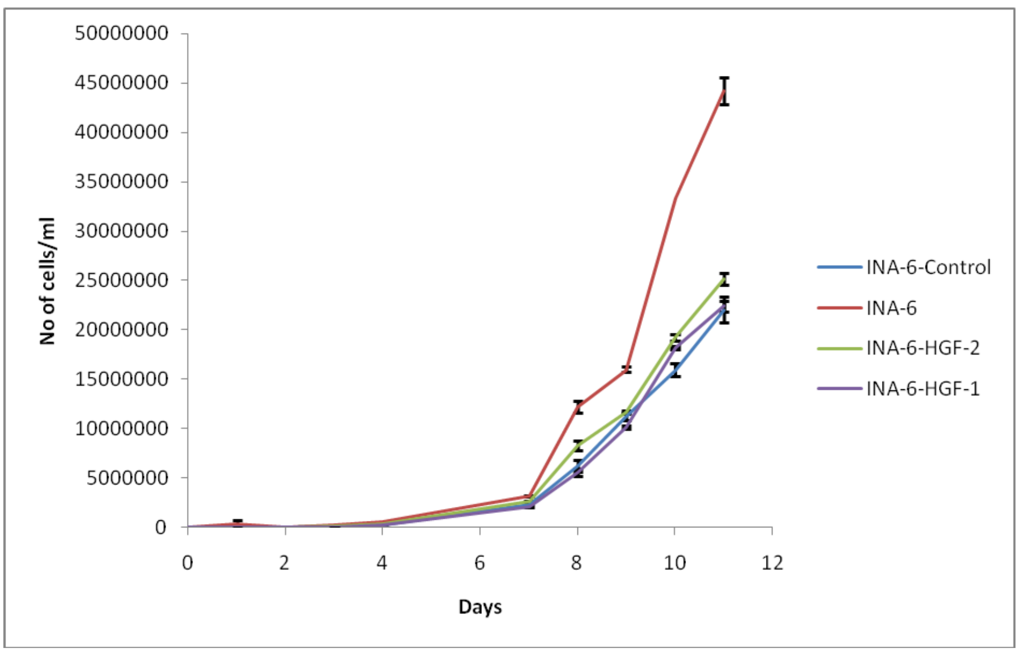

Fig 4: Growth curves of INA-6, INA-6-HGF-1, INA-6-HGF-2 and INA-6-control. Cell were seeded with equal density (50000 cells/ml of culture media) of each cell line at the start of experiment and growth was monitored by counting cells every 24 h. One biological experiment with two replica (± SD). One representative experiment out of three is shown.

any growth advantages.

DISCUSSION

RNAi is generally used in biomedical research to study what happens when a gene expression is blocked or reduced. On the otherhand, Stable cell lines with specific gene over- expression are very helpful in revealing gene functions.

The aim of this study was to analyze the INA-6 cell lines (INA-6-HGF-1 and INA-6-HGF-2) stably transduced with HGF expression plasmid. These cell lines were previously established in the myeloma group. The HGF overexpression was analyzed in these cell lines both at RNA level and protein level (Figure1-2). The HGF-transduced cells were also analyzed for the amount of secreted HGF protein (Figure 3). The results showed high expression of HGF mRNA in the HGF transduced cells. No HGF mRNA was produced in control cell lines (INA-6 and INA-6 control). The HGF mRNA levels in these cells were very high, compared to other HGF producing myeloma cell lines. On Western Blot, typical three bands of HGF protein were found in the HGF producing cell lines JJN-3 and ANBL-6. No HGF protein was observed in INA-6 control cells. Three bands in HGF overexpressing cell lines were expected. But unexpectedly only the band, approximately the size of the α-chain of the HGF protein, in these HGF over expressing cell lines was detected (Figure 2). It was also noticed that α chain band was slightly smaller in size than that found in JJN-3-sh non target, JJN-3 Turbo GFP and ANBL-6 (Figure 2). The reason for this discrepancy is not clear at this level. One possible explanation could be that there is a mutation in the HGF gene sequence, leading to a premature stop codon in the translation process. This will give a truncated HGF protein. A sequencing of the complete HGF gene sequence in the expression plasmid used in the stably transfection of the cells, should be performed to verify or null out this hypothesis.

Secretion of HGF protein in these transduced cell lines was also proved by ELISA. However the secretion of this protein was not that high as in HGF producing cell lines ANBL-6 and JJN-3 (Figure 4). So compared to the high level of mRNA, the secreted levels of HGF protein were rather low. The reason for this is not easy to determine at this stage. However, it could be, as discussed above, that a truncated HGF protein is produced, and this is only partly recognized by the HGF antibody in the ELISA assay.

As discussed earlier, HGF is an important factor for proliferation and survival of myeloma cell.[15] It was therefore interesting to know if HGF over expression give any growth advantage to these HGF producing INA-6 cell lines as compared to non HGF producing cells INA-6 and INA-6-control. We therefore analyzed the growth curves for the transducer cells (INA-6-HGF-1, INA-6-HGF-2). Our result showed that the HGF overexpressing cells did not have any significant growth advantage over the control cell lines (Figure 4). However all the transduced cells seem to grow slower than the untransduced INA-6 cell line ((Figure 4).

CONCLUSIONS

In this work INA-6 cell lines were over expressed using HGF gene. Though the HGF protein was not produced all in correct form, still this work demonstrate that myeloma cell lines can be genetically engineered for the expression of different proteins serving as heterlogous system for research purpose.

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