Figures

Part of introduction

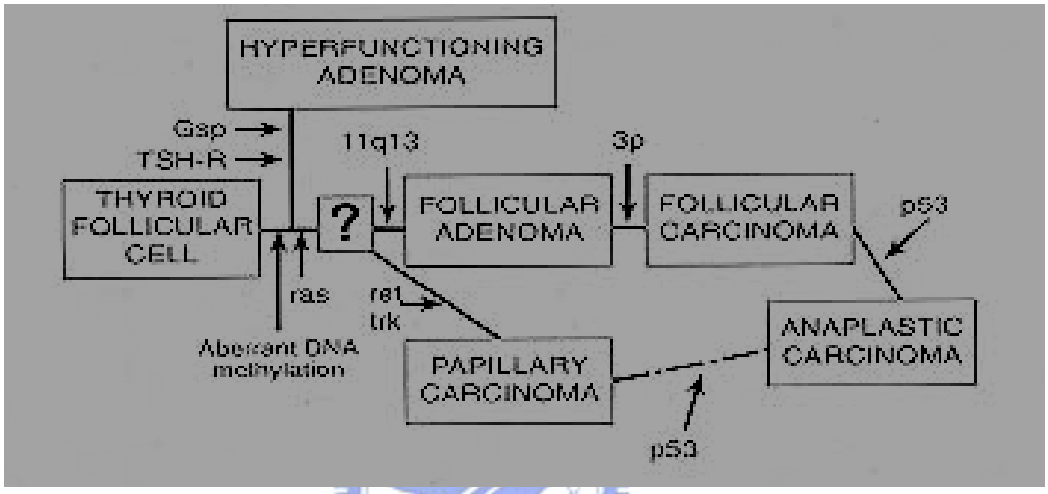


Figure 1. Possible role of oncogene activation, receptor, G-protein mutation, or tumor supressor gene deletion in the induction of thyroid carcinoma. (by James A Fagin, M.D.)

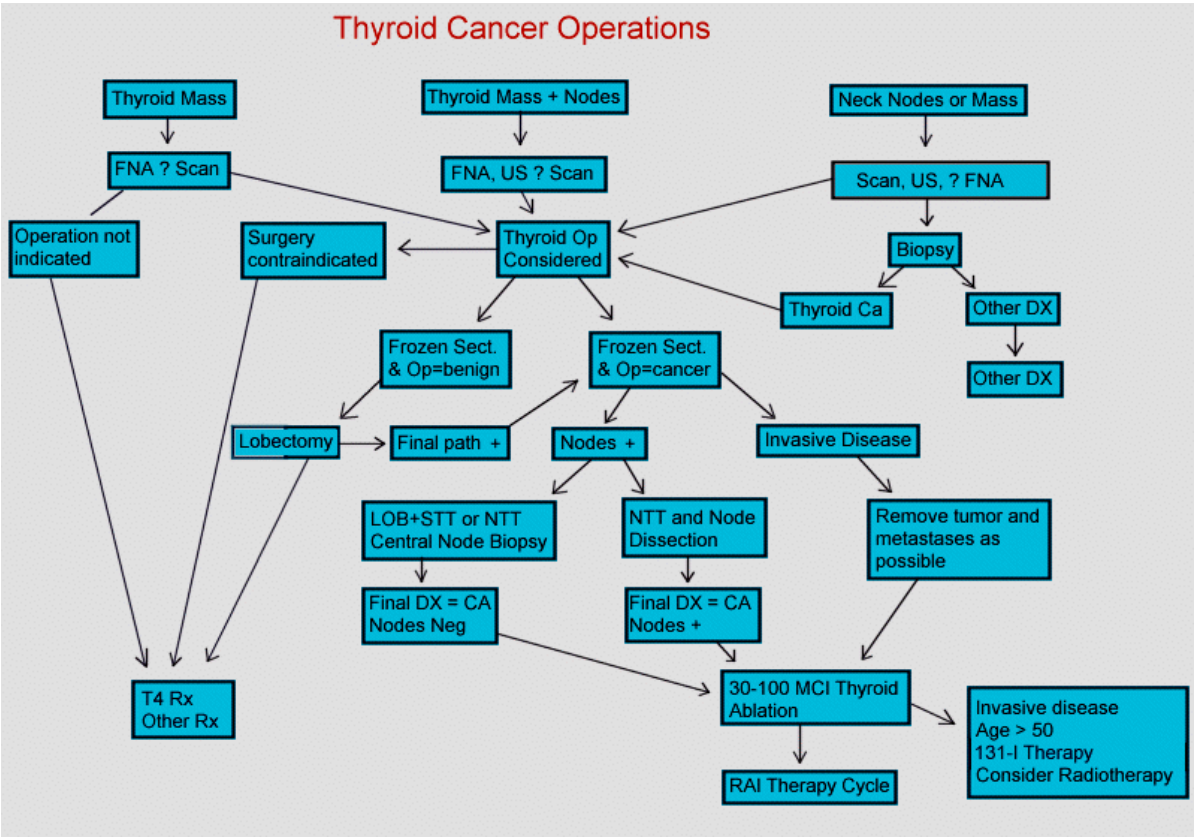


Fig. 2 The detection methods of thyroid cancer. (by Furio Pacini, M.D., and Leslie J. De Groot, M.D.)

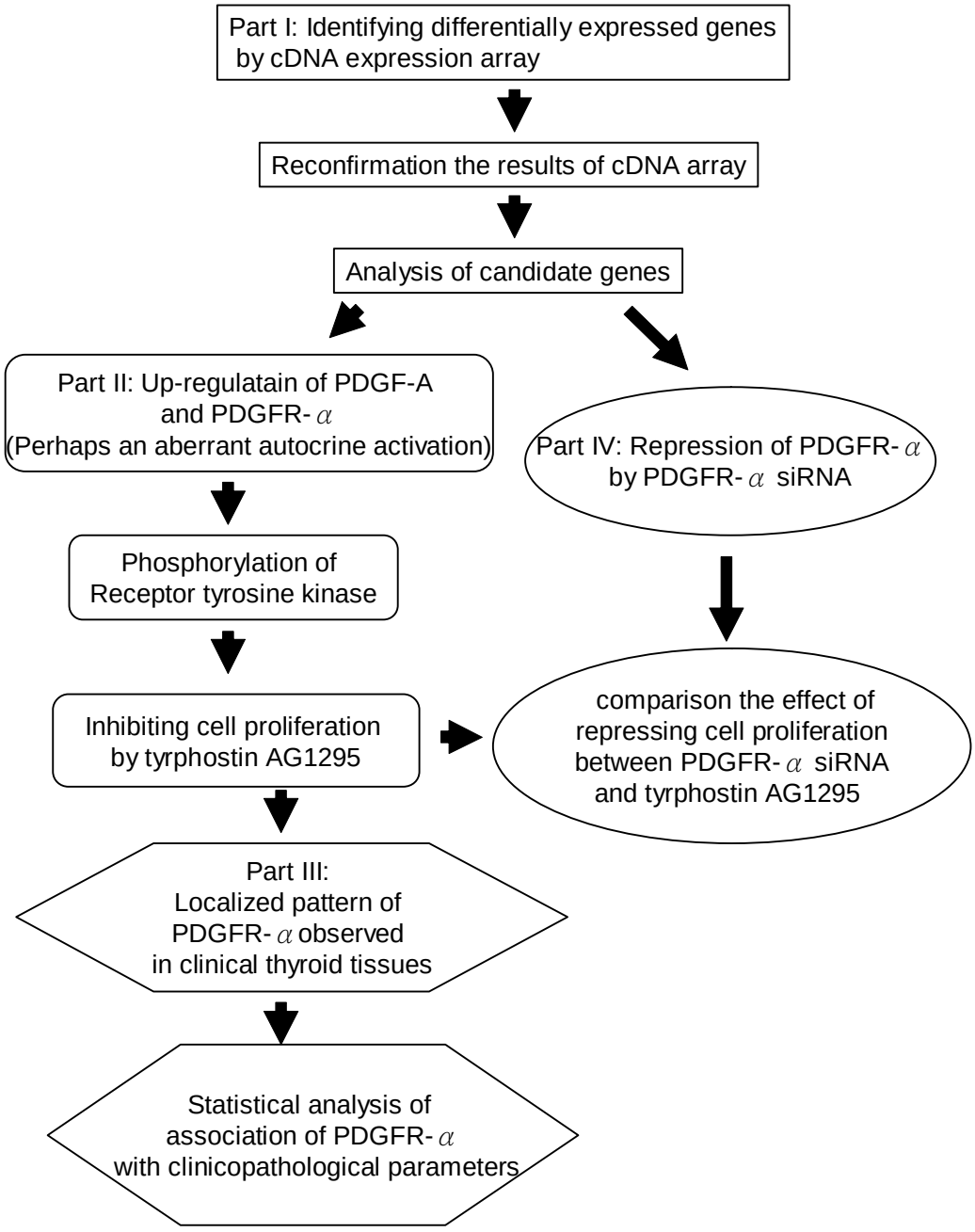


Fig 3. The proposal of studying the PDGFR-α mechanism in thyroid carcinogenesis.

Part 1.

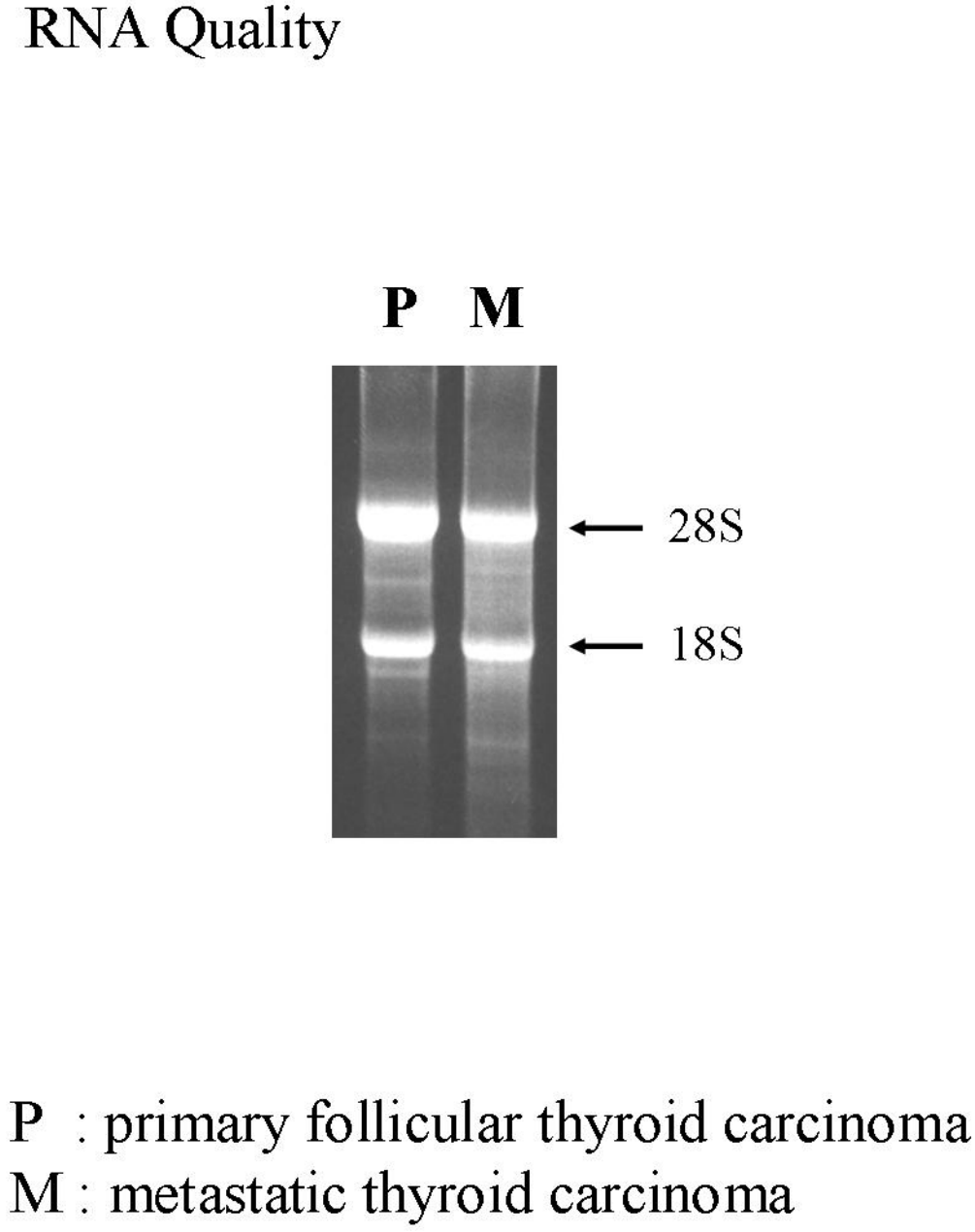


Figure 1. The quality of total RNA isolated from primary and metastatic follicular thyroid carcinoma. The agarose electrophoresis contained 10 μg total RNA per lane.

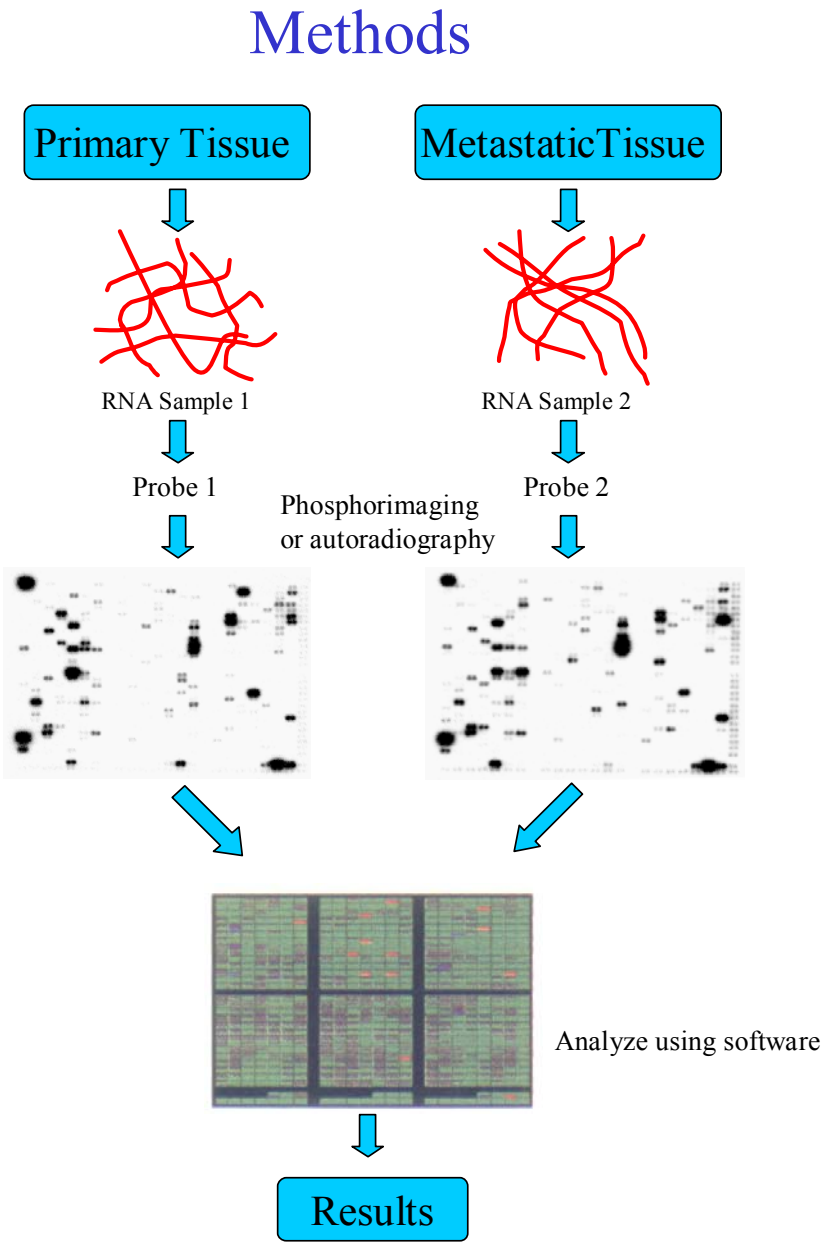


Figure 2. **The procedure of cDNA array experiment in metastatic and primary follicular thyroid carcinoma.**

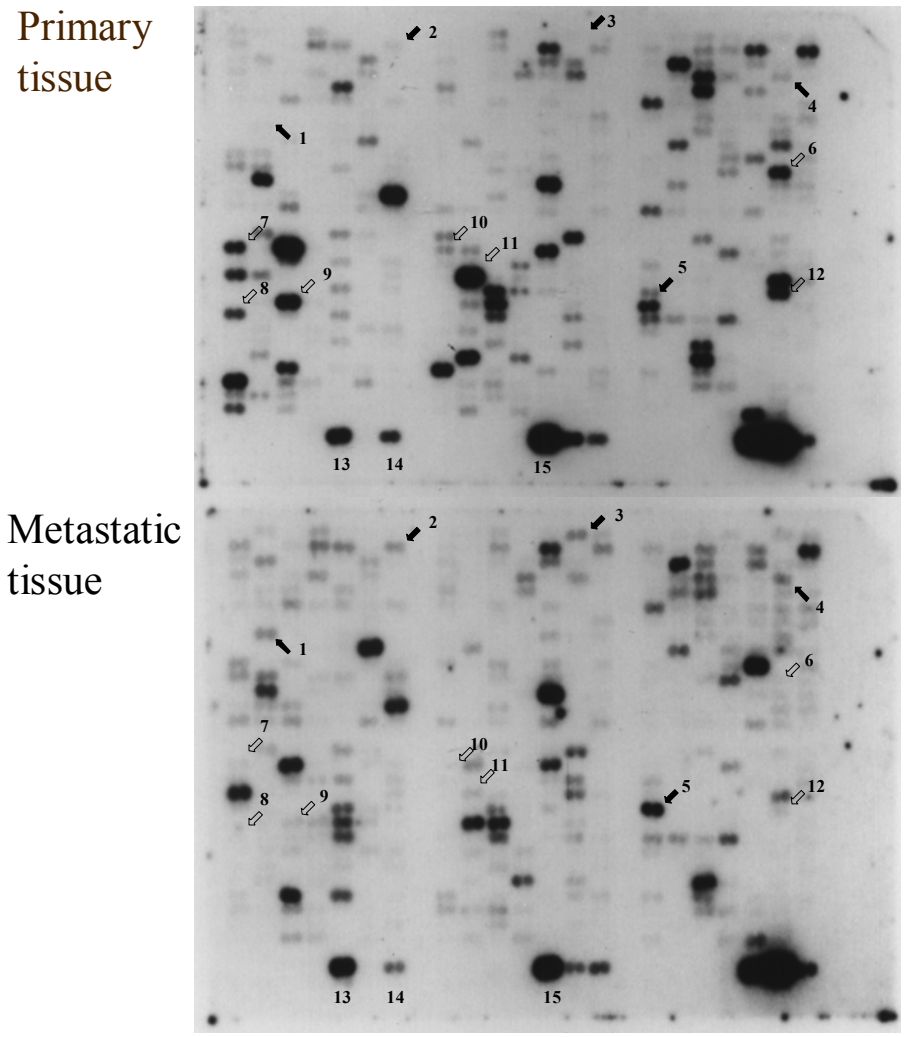


Figure 3. Expression pattern of genes in follicular thyroid carcinoma and its pair metastatic tissue. The top and bottom panels represent the expression array membrane hybridized with cDNA from thyroid carcinoma and metastatic tissues, respectively. Solid arrows indicate genes that are over-expressed in metastatic tissue, and open arrows indicates genes that are over-expressed in primary thyroid carcinoma. Some representative genes are designated by number; 1, *CDC2-related protein kinase*; 2, *cytokeratin 18*; 3, *c-fos*; 4. *growth hormone-binding protein (IGFBP)*; 5, *heparin-binding vascular endothelial growth factor*. 6, *PDGF-alpha receptor*; 7, *byglycan*; 8, *collagen type I*; 9, *fibronectin*; 10, *gelatinase A*; 11, *mitogen-inducible gene*; 12, *platelet-derived growth factor A*. Housekeeping genes that are expressed at similar levels in both panels are designated as 13, *ubiquitin*; 14, *hypoxanthine-quanine phosphoribosyltransferase*; 15, *liver glyceraldehydes 3-phosphate dehydrogenase (GAPDH)*.

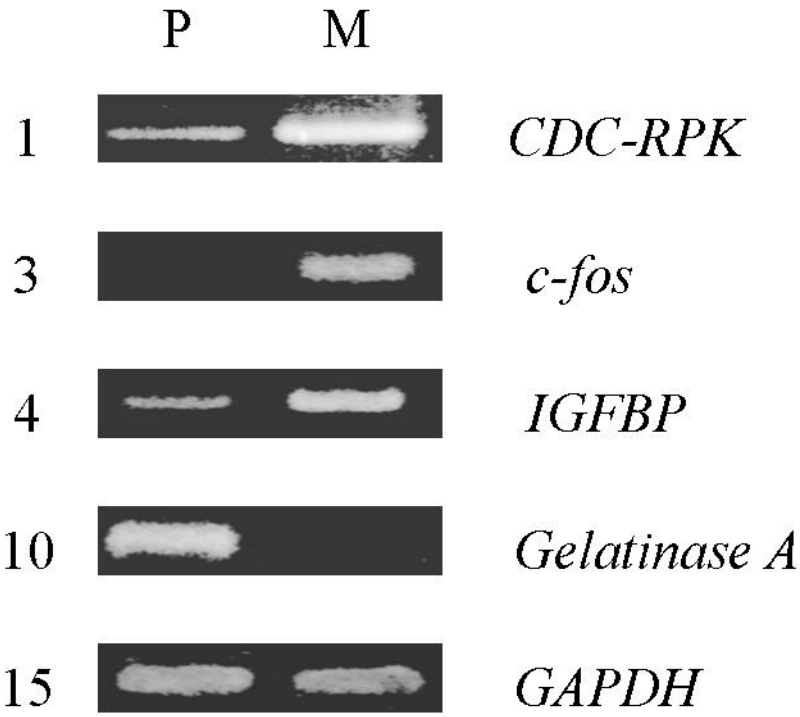


Figure 4. Reverse transcription-polymerase chain reaction substantiating the consistency of the cDNA array results. Lane P represents primary follicular carcinoma tissue and lane M represents metastatic tissue. Corresponding locations within the cDNA array illustrated in Figure 3 are provided for 1, *CDC2-related protein kinase*; 3, *c-fos*; 4, *IGFBP*; 10,*Gelatinase A*; 15, *GAPDH*. The *GAPDH* represents a control for loading variance.

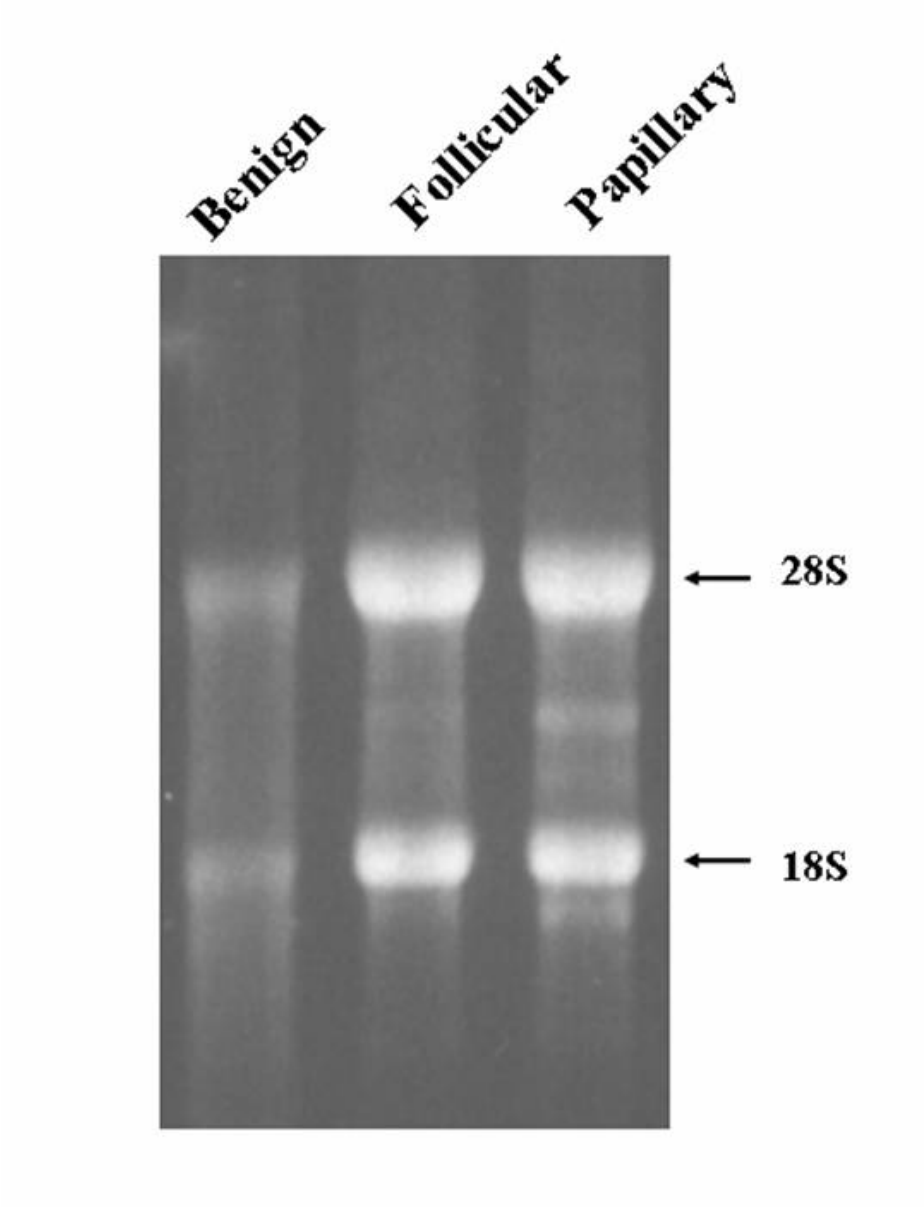


Figure 5. The quality of total RNA isolated from tissue derived from nodular hyperplasia, follicular thyroid carcinoma cell line (CGTH W-1) and papillary thyroid carcinoma cell line (CGTH W-3). The agarose electrophoresis contained 10 μg total RNA per lane.

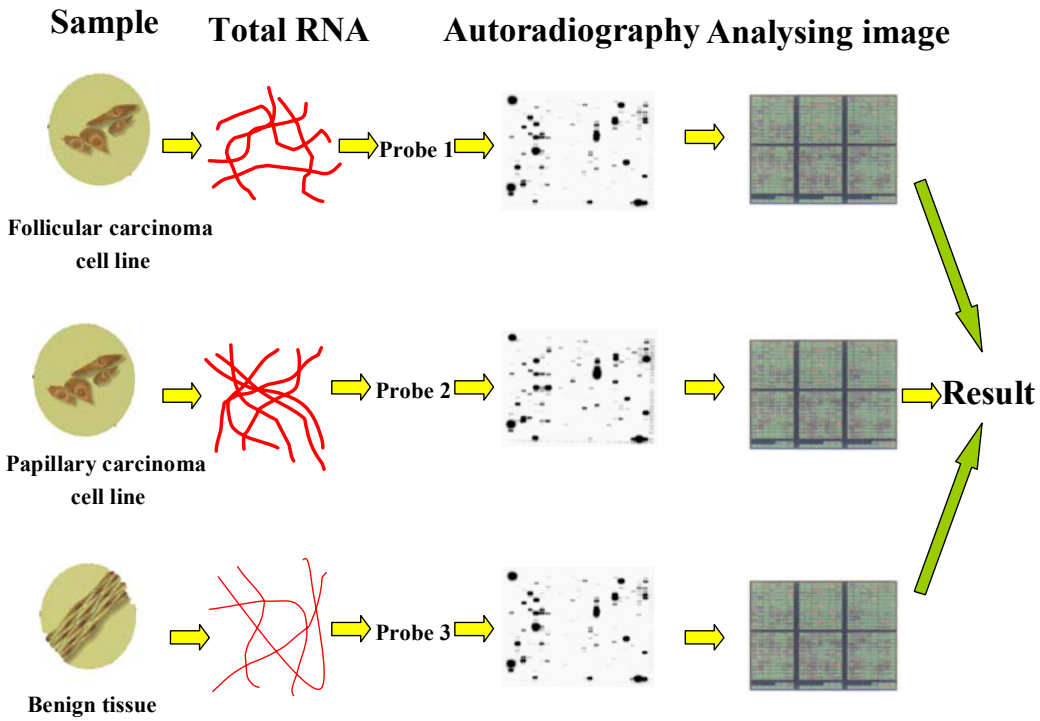
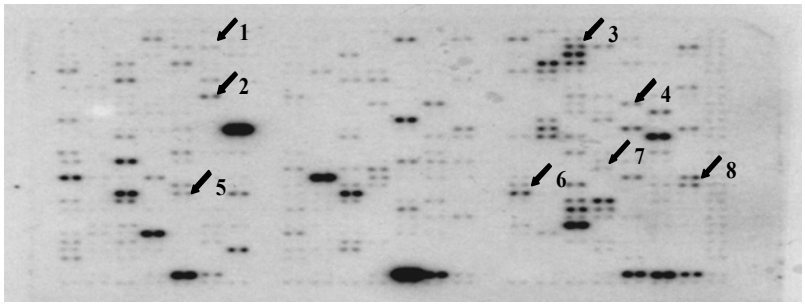
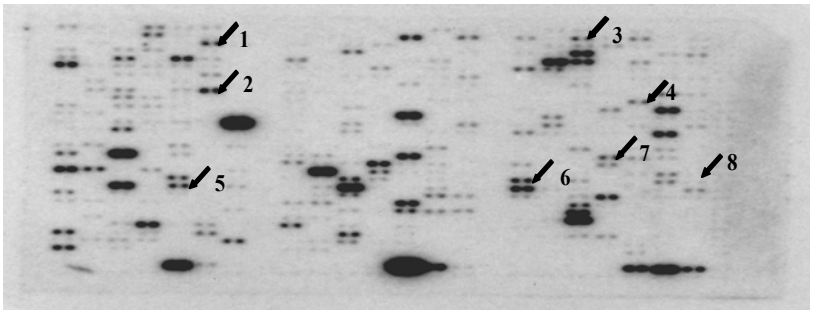


Figure 6. The procedure of cDNA array experiment in nodular hyperplasia (benign tissue), follicular thyroid carcinoma cell line (CGTH W-1) and papillary thyroid carcinoma cell line (CGTH W-3).

Follicular thyroid carcinoma cell line



Papillary thyroid carcinoma cell line



Nodular hyperplasia

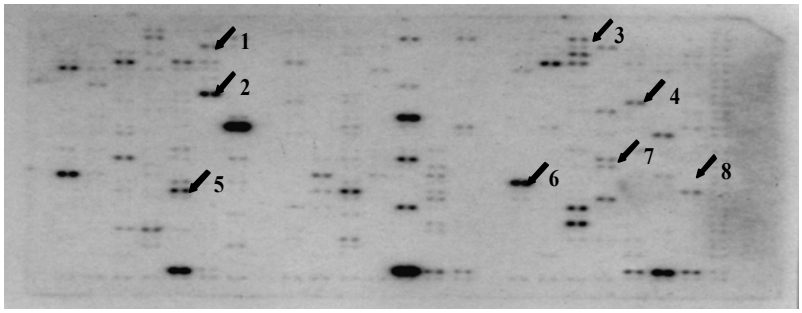


Figure 7. Expression pattern of genes in follicular thyroid carcinoma cell line, papillary carcinoma cell line and nodular hyperplasia. Differential hybridization of three identical human cDNA expression arrays was performed as described in Materials and Mehtods. Solid arrows indicates genes that are differentially expressed in follicular thyroid carcinoma cell line as compared with papillary thyroid carcinoma cell line and nodular hyperplasia. Some representative genes are designated by number; 1, *Growth factor receptor-bound protein 2 (GRB2) isoform*; 2, *c-myc binding protein MM-1*; 3, *Cytosolic superoxide dismutase 1(SOD1)*; 4, *fau*; 5, *CD9 antigen*; 6, *Vascular endothelial growth factor precursor (VEGF)*; 7, *Interferon gamma antagonist (INF-gamma antagonist)*; 8, *CD30 ligand(CD30L)*.

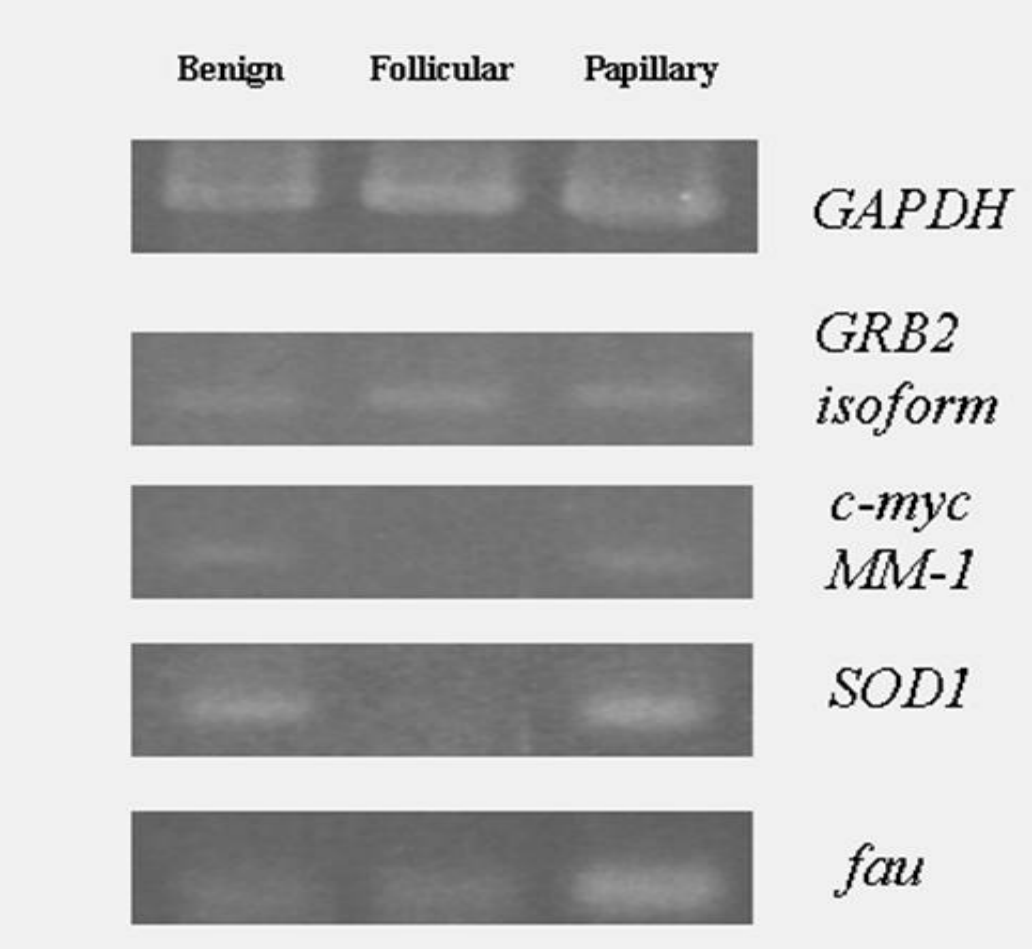


Figure 8. Reverse transcription-polymerase chain reaction confirming the results of the cDNA array. The GAPDH represents a control for loading variance.

Part 2.

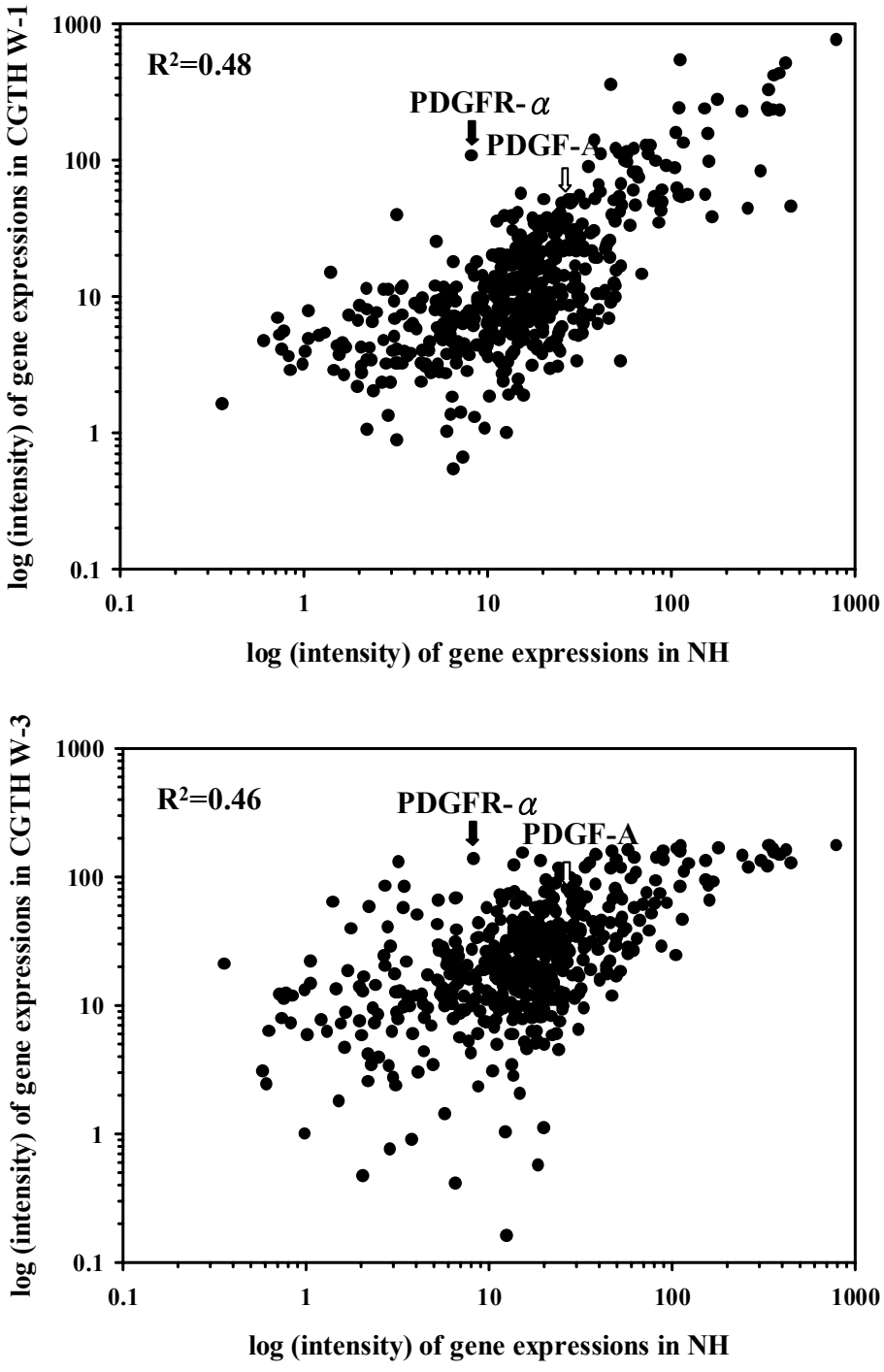


Figure 1. The list of intensity obtained from gene expressions of human cDNA expression arrays under the thyroid carcinoma cell lines and nodular hyperplasia. Scatter plots of the calibrated intensity (log scale) compared to give a correlation coefficient between CGTH W-1 cell and nodular hyperplasia (upper plot) or between CGTH W-3 cell and nodular hyperplasia (lower plot) were shown.

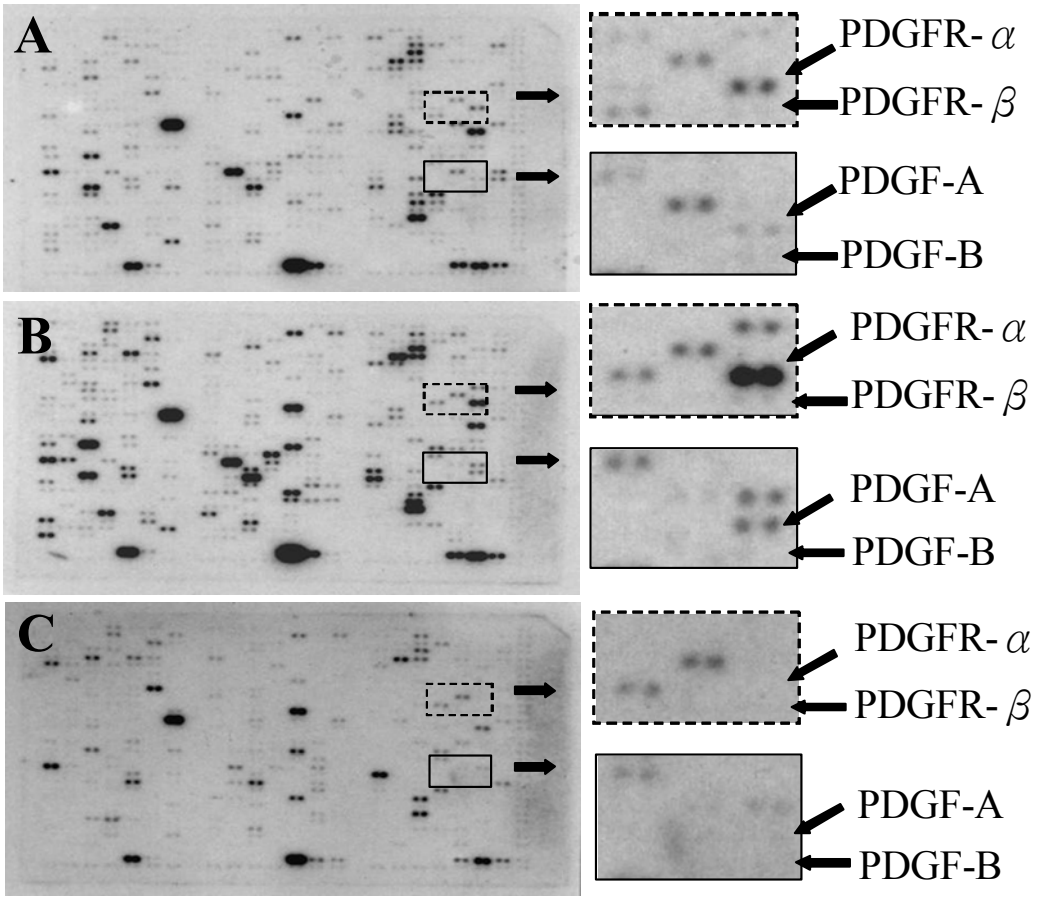


Figure 2. Expression pattern of genes in follicular thyroid carcinoma cell line, papillary thyroid carcinoma cell line, and nodular hyperplasia. Differential hybridization of three identical human cDNA expression arrays was performed as described in Materials and Methods. The top, middle, and bottom panels represent the expression array membrane hybridized with cDNA from (A)follicular thyroid carcinoma cell line, (B)papillary thyroid carcinoma cell line, and (C)nodular hyperplasia, respectively. Solid arrows indicate the gene expression level of PDGF-A, PDGF-B, PDGFR-α, and PDGFR-β.

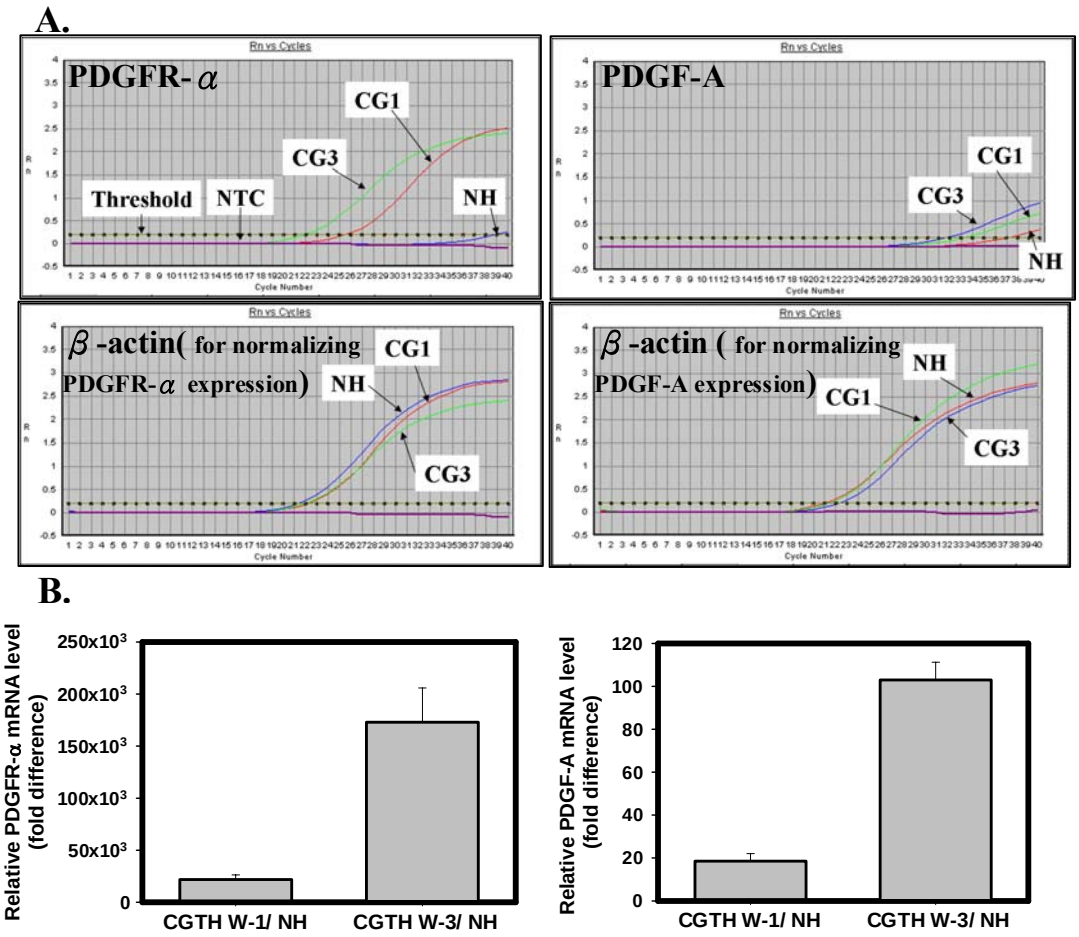


Figure 3. Quantitative PCR reconfirm the tendency towards up-regulation expression of PDGF-A and PDGFR-α in CGTH W-1 and CGTH W-3 cell lines. (A) An example of a represent PDGF-A, PDGFR-α and β-actin amplification plot of the quantitative PCR. The Y-axis shows the fluorescence calculated by subtracting the background fluorescence (Rn) whereas the X-axis shows the number of PCR cycles. A higher number of PCR cycles is required to pass the fixed threshold in tissue sample from nodular hyperplasia (NH). However, samples from CGTH W-1 (CG1) or CGTH W-3 (CG3) cell lines decrease the number of cycles required to pass the threshold , which indicate that more PDGF-A and PDGFR-α gene is present in these samples. (B) The average of three independent experiments is shown, error bars indicate standard deviation. These values were normalizing according to the expression data of the housekeeping gene, β-actin.

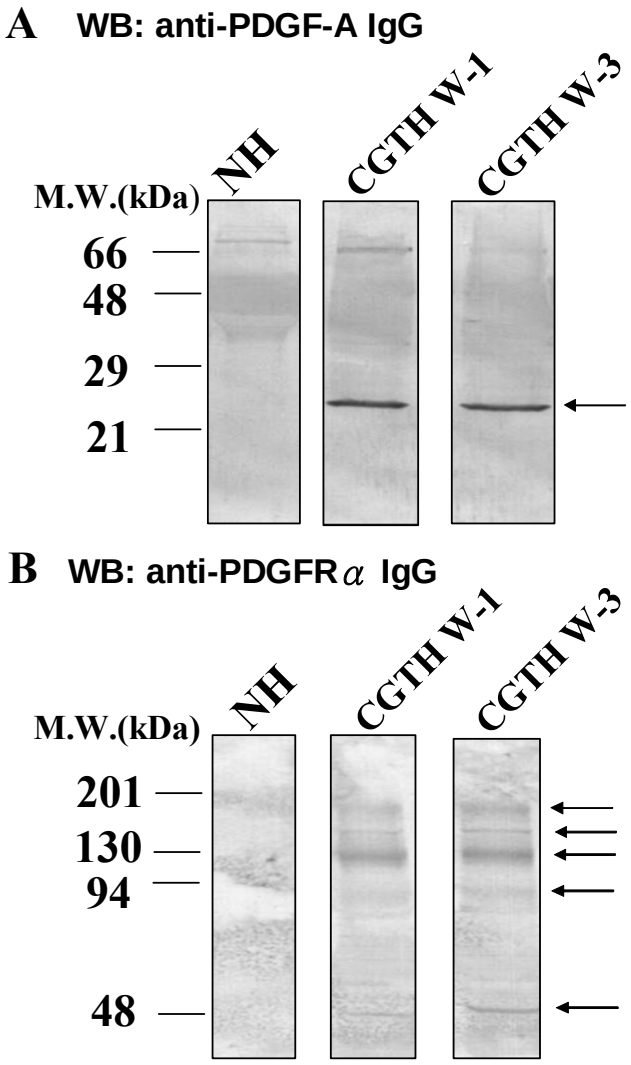


Figure 4. Detection of PDGF-A and PDGFR-α by western blotting. (4A) The approximate 26 kDa PDGF-A protein was detected with mouse monoclonal IgG_{2b} anti-PDGF-A in nodular hyperplasia(NH), follicular thyroid carcinoma cell line (CGTH W-1), and papillary thyroid carcinoma cell line (CGTH W-3). (4B) The approximate 180, 156, 130, 90 and 52 kDa protein were detected with rabbit polyclonal IgG anti-PDGFRα in nodular hyperplasia(NH), follicular thyroid carcinoma cell line (CGTH W-1), and papillary thyroid carcinoma cell line (CGTH W-3).

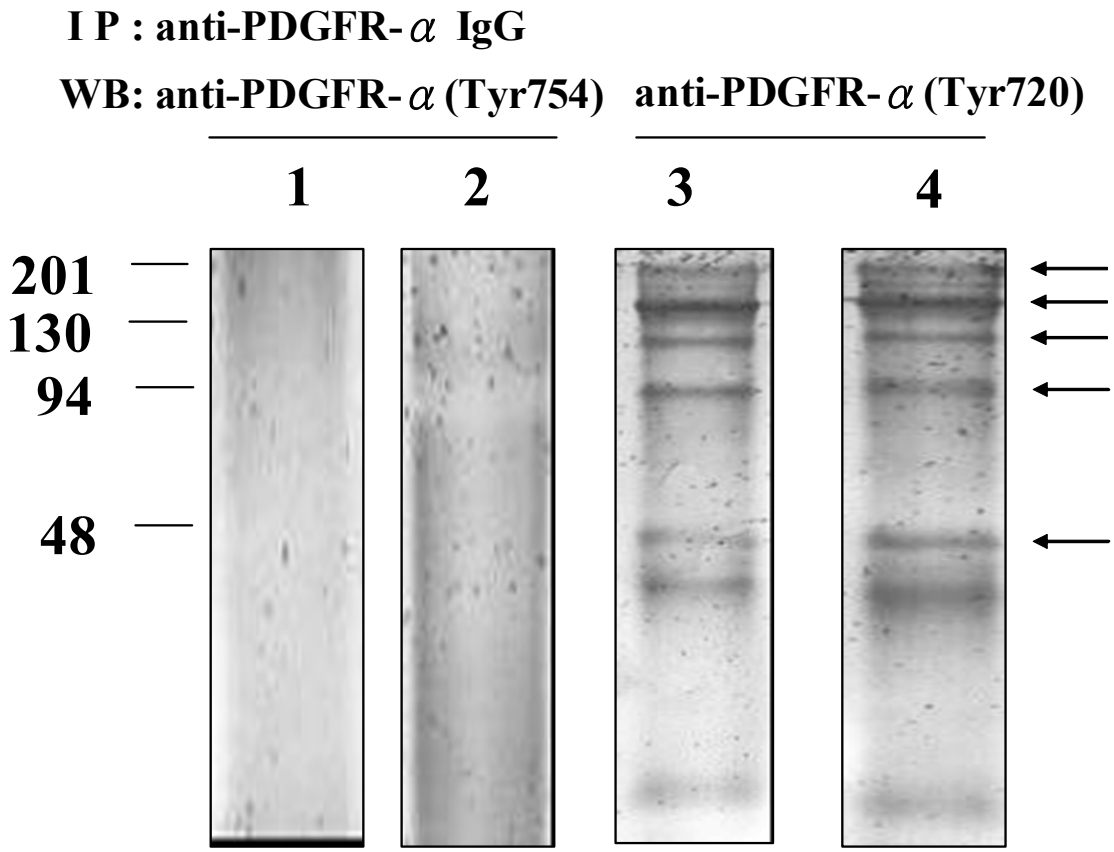


Figure 5. Detection of phosphorylated PDGFR-α with immunoprecipitaion followed by western blotting. The rabbit polyclonal IgG anti-PDGFRα was used to immunoprecipitate PDGFR-α protein expressed in CGTH W-1(lane 1 and 3) and CGTH W-3 (lane 2 and 4). Immunoprecipitates were subjected to SDS-PAGE and immunoblotted with p-PDGFR-α(Tyr754) (lane 1 and 2) and p-PDGFR-α(Tyr720) (lane 3 and 4).

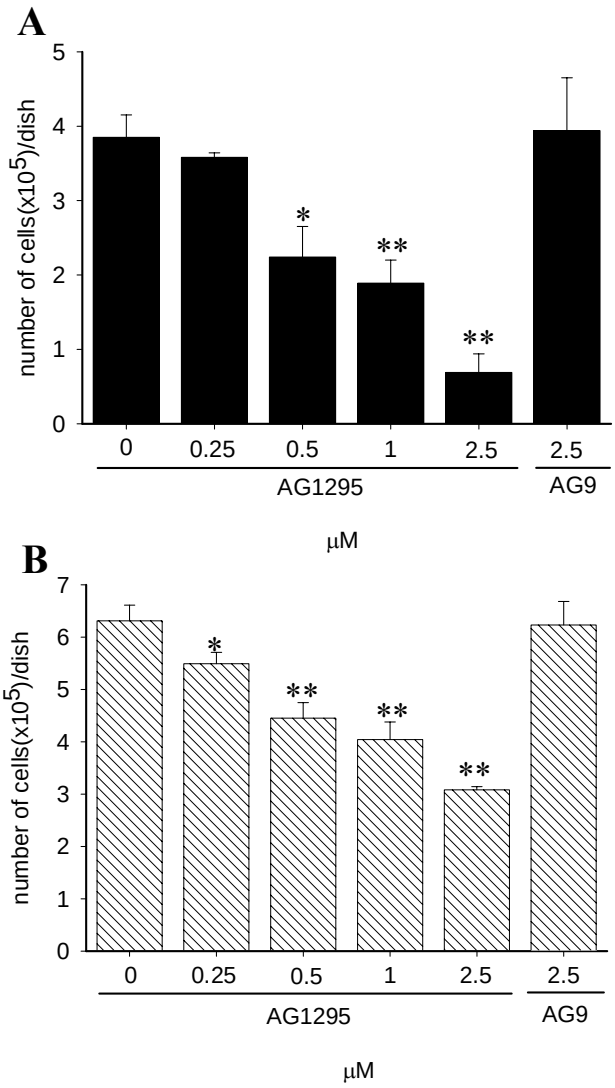


Figure 6. Effects of Tyrphostin A1 (AG9) and Tyrphostin AG1295 on proliferation of CGTH W-1 and CGTH W-3 cell lines. Approximately 2.5 x 10^4 cells were seeded in RPMI-1640 medium with 1% fetal calf serum in triplicate dishes and grown in the presence of selective inhibitor for PDGF-receptor (0, 0.25, 0.5, 1, and 2.5 μM Tyrphostin AG1295) and negative control (2.5 μM Tyrphostin A1). (A) CGTH W-1. (B) CGTH W-3. Data are expressed as mean $\pm$ S.D. The *t* test was used to determine whether two experimental values were significantly different. *p<0.05 and **p<0.001 compared to the results of 0 μM Tyrphostin AG1295 treatment.

Part 3.

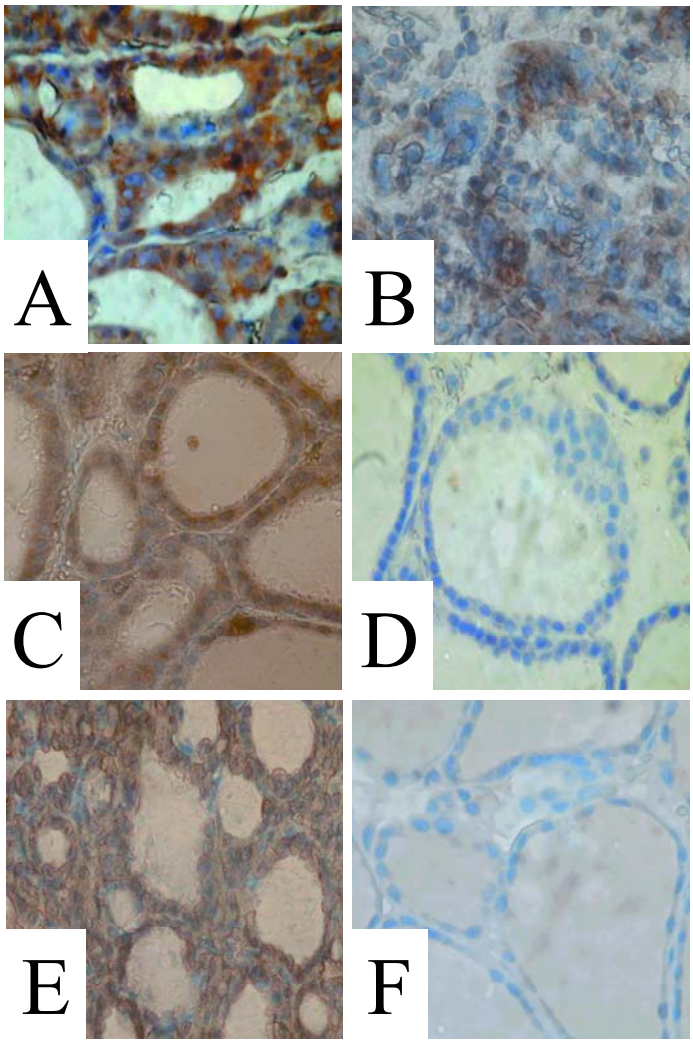


Figure 1. Immunohistochemical detection of PDGFR-α in (A) follicular thyroid carcinoma (clinical stage III), (B) follicular thyroid carcinoma with bone metastases (clinical stage IV), (C) follicular thyroid carcinoma (clinical stage II) , (D) thyroid nodular hyperplasia. Homogeneous positive staining of β-actin is seen in FTC clinical stage I specimen(E). There was no specific staining on the FTC specimen with normal goat serum IgG (F). Original magnification, x 200. Various density of immunoperoxidase staining is seen in brown by using 3,3' diaminobezidene (DAB) substrate. Nucleus stained in hematoxylin is shown in blue.

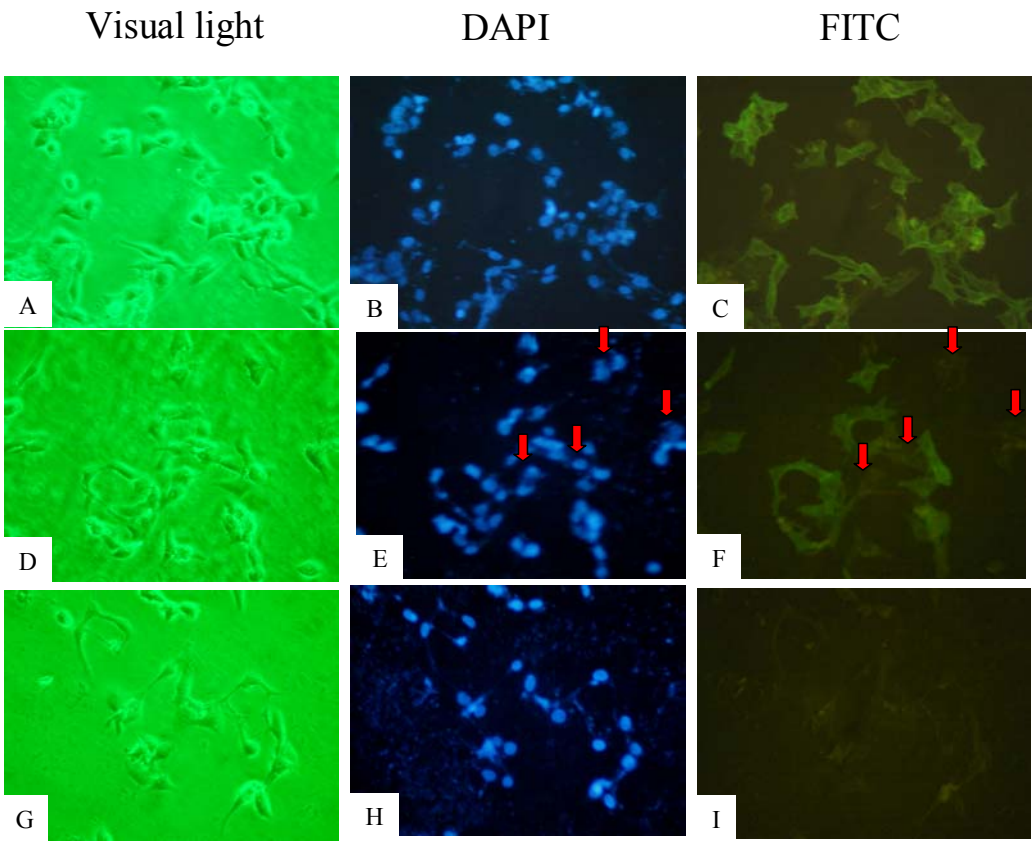
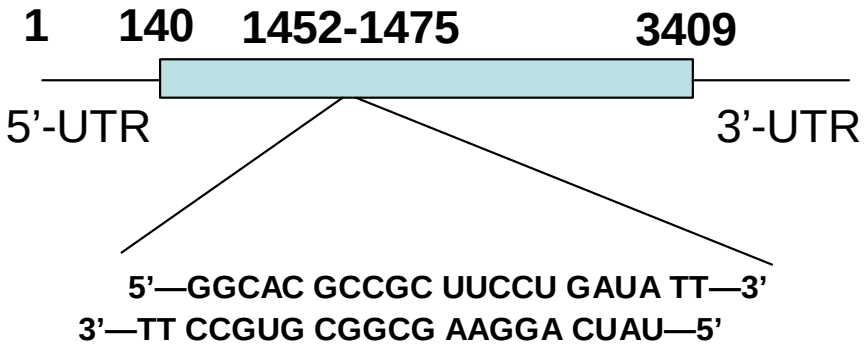


Figure 2. Immunofluorescence cell staining of PDGFR-α in follicular thyroid cell line(CGTH W-1). CGTH W-1 cells were transfected with non-silencing siRNA (A, B, C), PDGFR-α siRNA at day 0 only (D, E, F) or PDGFR-α siRNA twice at day 0 and 2 (G, H, I). The fourth day after transfection, cells were fixed and stained with 4',6'-diamidino-2-phenylindole(DAPI) (blue color) for cell nuclei and with PDGFR-α specific antibody and secondary antibody conjugated FITC(green color). The arrows in the middle panel indicate the cells repressed the expression of PDGFR-α.

Part 4.

a. **PDGFR-α siRNA**



b. **Control siRNA (non-silencing)**

5'—UUCUC CGAAC GUGUC ACGU TT—3'
3'—TT AAGAG GCUUG CACAG UGCA—5'

Figure 1. Schematic representation of PDGFR-α siRNA and control siRNA. (a) The 1452-1475 nucleotide region of the *PDGFR-α* (accession number: M21574) was targeted by PDGFR-α siRNAs. Schematic structure of the PDGFR-α siRNA that sense and antisense sequence of both RNA strands were annealed to dsRNA are shown. (b) To control the specificity of the RNAi reaction, control siRNA was used and shown.

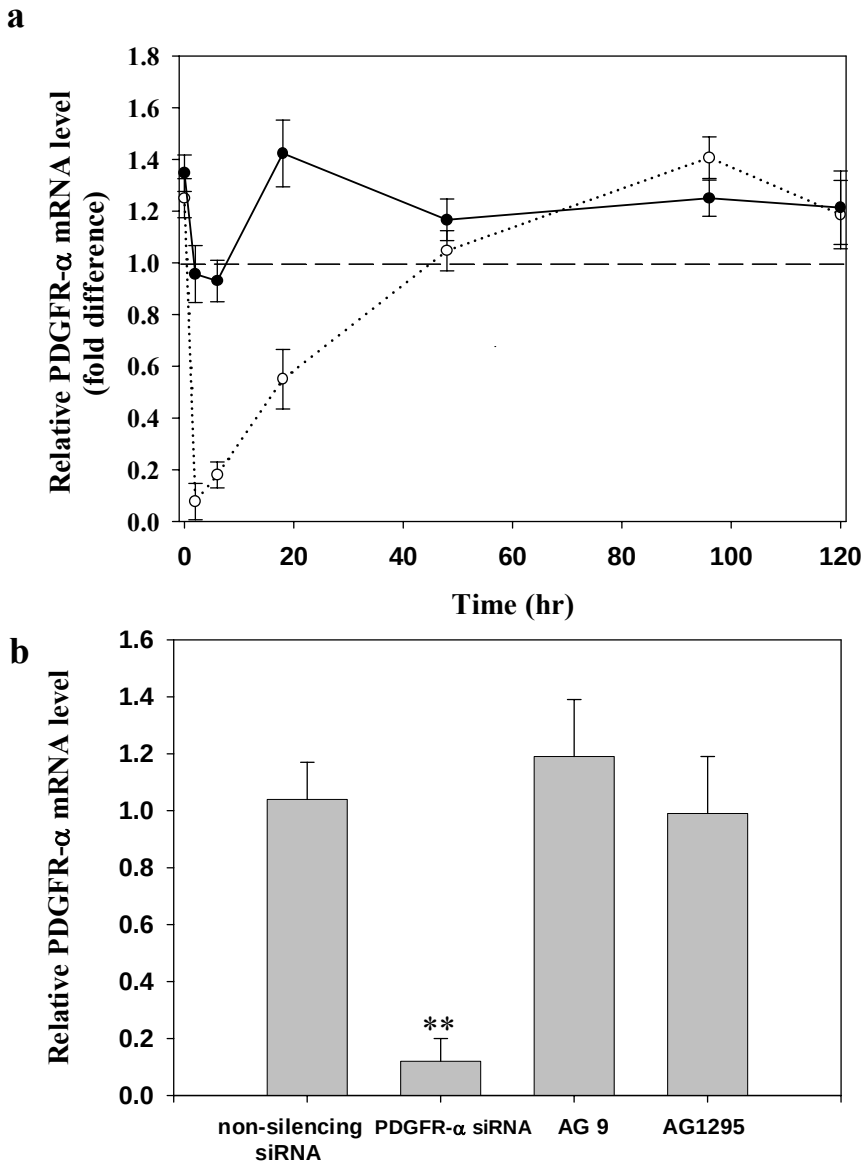
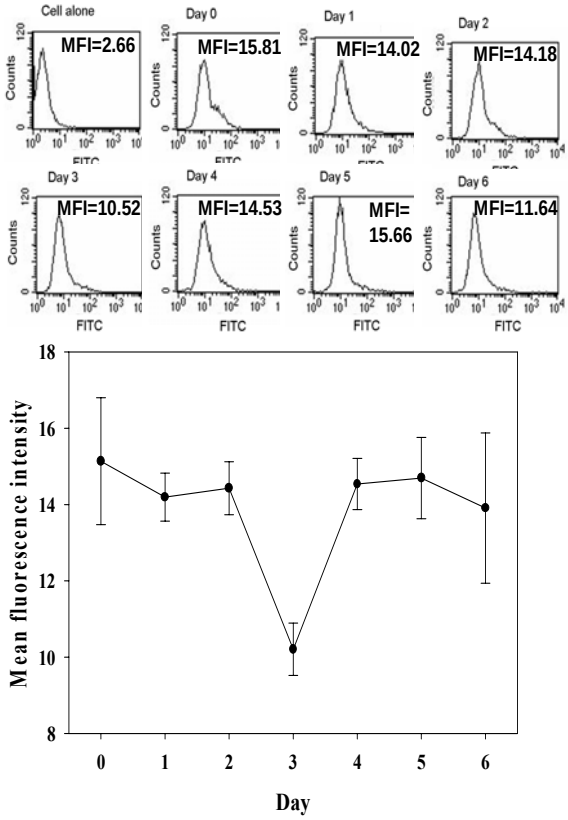
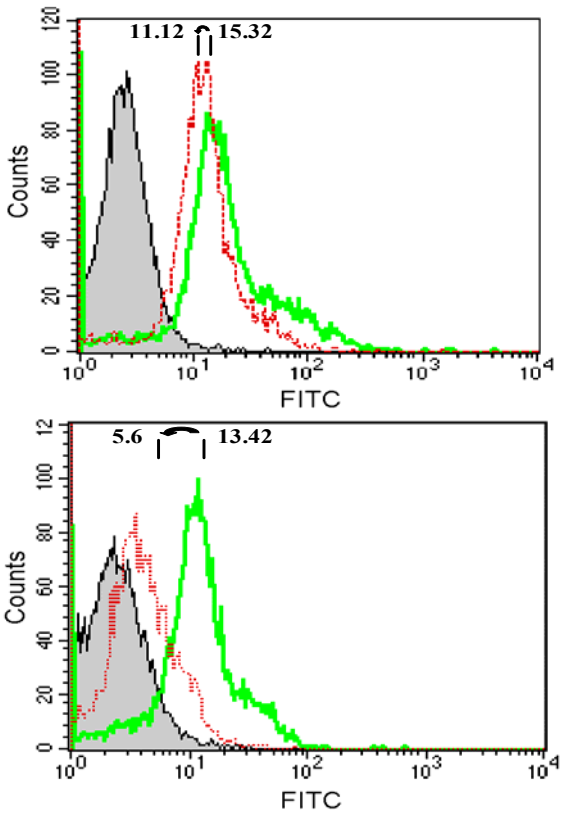


Figure 2. PDGFR-α siRNA leads to promote cleavage of the cellular *PDGFR-α* mRNA. (a) CGTH W-1 cells were tranfected with PDGFR-α siRNA or control siRNA. Cells were subjected to quantitative PCR assay until 120hr post-transfection. The relative *PDGFR-α* mRNA levels were shown as the RNA level of transfecting with PDGFR-α siRNA (open circle) and non-silencing siRNA (solid circle) compared with those of mock transfection and represented the copy number of *PDGFR-*α mRNA from CGTH W-1 cells transfected with siRNA divided by that from CGTH W-1 cells performed mock transfection normalized to β*-actin* (at each time point). (b)The mean values of relative *PDGFR-α* mRNA level for three experiments $\pm$ S.D were shown, including non-silencing siRNA, PDGFR-α siRNA, tyrphostin AG 9 and tyrphostin AG1295. **, student *t* test show $p<0.001$ significantly different.

Figure 3a


Cell alone
MFI=2.66
Day 0
MFI=15.81
Day 1
MFI=14.02
Day 2
MFI=14.18
Day 3
MFI=10.52
Day 4
MFI=14.53
Day 5
MFI=
15.66
Day 6
MFI=11.64
Counts
FITC
Mean fluorescence intensity
Day

3b


11.12
15.32
5.6
13.42
Counts
FITC

3c

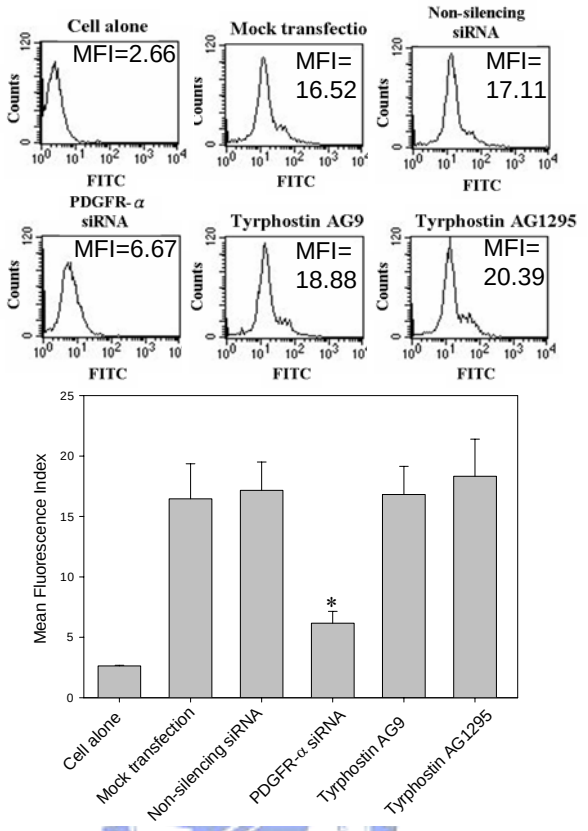


Figure 3. PDGFR-α siRNA reduce the PDGFR-α protein expression. (a) Flow cytometry (FCM) analysis of PDGFR-α expression in CGTH W-1 cells transfected with PDGFR-α siRNA for 6 days post-transfection. (b) FCM profiles of CGTH W-1 cells transfected once with non-silencing siRNA (upper panel; green; MFI=15.32) and PDGFR-α siRNA (red; MFI=11.12). Control uninfected cells are indicated by solid gray section (MFI=2.62).The profile of CGTH W-1 cells transfected twice with non-silencing siRNA (green; MFI=13.42) and PDGFR-α siRNA (red; MFI=5.6) were shown in lower panel. Simultaneously, control uninfected cells are indicated by solid gray section (MFI=2.53). (c) FCM analysis, cells were treated with mock transfection, non-silencing siRNA, PDGFR-α siRNA, tyrphostin AG9 and tyrphostin AG1295. The mean fluorescence indexes of PDGFR-α protein for three experiments $\pm$ S.D. were shown. *, student *t* test show $p<0.05$ significantly different.

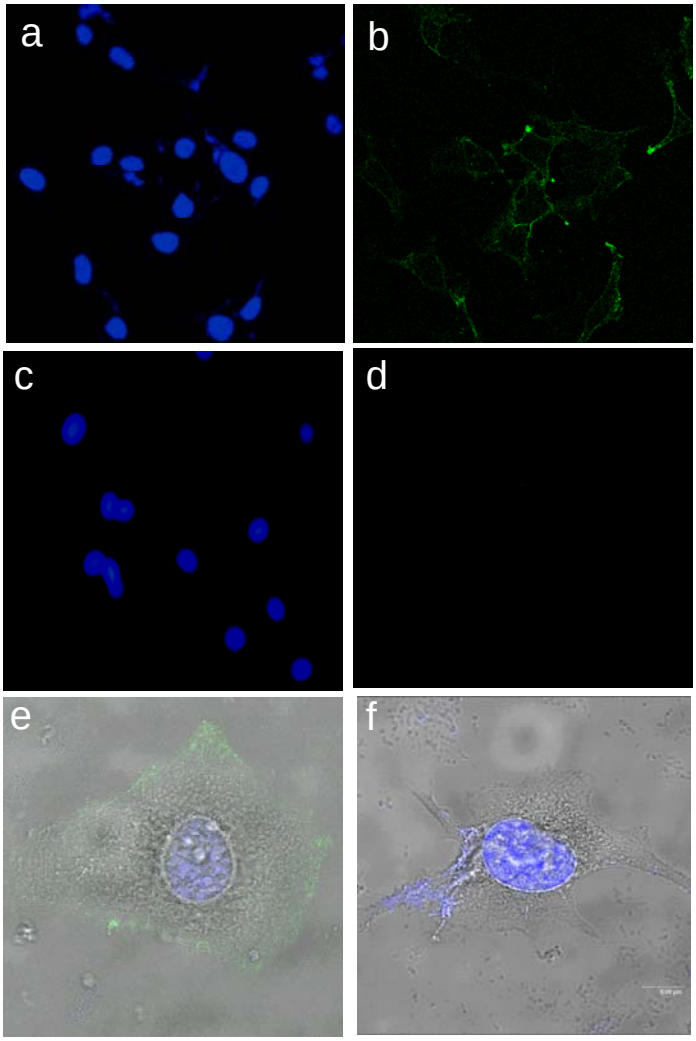


Figure 4. Immunofluorescence localization of PDGFR-α in CGTH W-1 cells. CGTH W-1 cells were transfected with PDGFR-α siRNA (**c, d and f**) and with non-silencing siRNA (**a, b and e**). **A** and **c**, DAPI staining of nuclear chromatin(blue color); **b** and **d** ,staining with PDGFR-α specific antibody and secondary antibody conjugated FITC(green color). **E** and **f**, showing cell morphologic appearance and nuclear staining (blue color), moreover **e**, showing membrane staining (green color); and **f**, showing that membrane staining was repressed by PDGFR-α siRNA. Images **a ,b ,c** and **d** were taken at a magnification of 200x, while images **e** and **f** were taken at a magnification of 1000x.

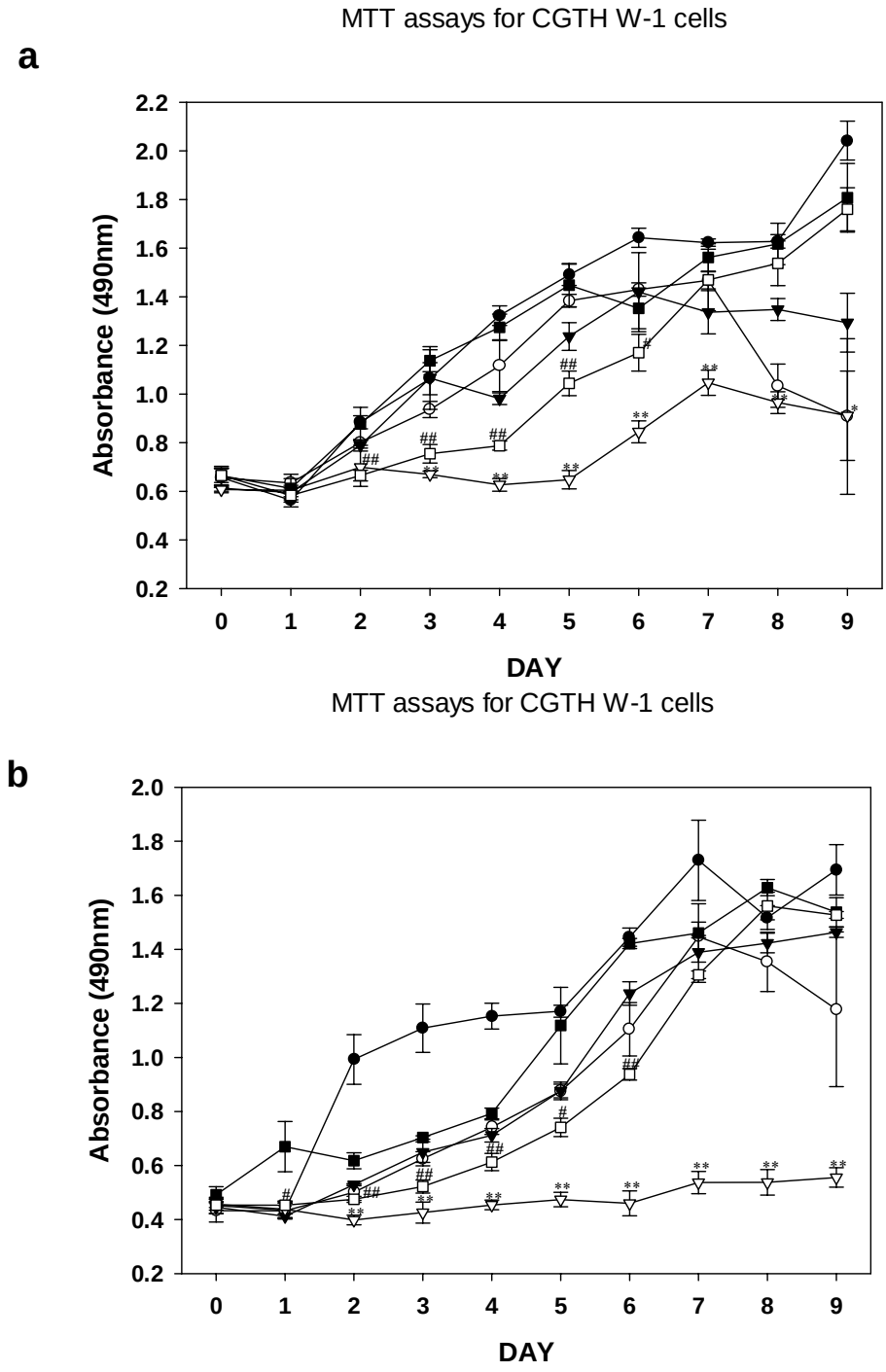


Figure 5. The inhibition efficacy of tyrphostin AG1295 and PDGFR-α siRNA on thyroid follicular carcinoma cell. A thousand (Figure 5a.) or a five hundred (Figure 5b.) CGTH W-1 cells were seeded in 96-well microtiter plates and then treated with different condition of tyrosine kinase inhibitor or genetic-specific siRNA, (—●— cell alone, —○— mock transfection, —▼— non-silencing siRNA, —▽— PDGFR-α siRNA, —■— tyrphostin AG9 and —□— tyrphostin AG1295).Cell proliferation was determined by the MTT assay. Results are the mean of three independent experiments. Bars represent S.D. * ,$^{\#}$ represent p <0.05 and **,$^{\#\#}$ represent p< 0.001.

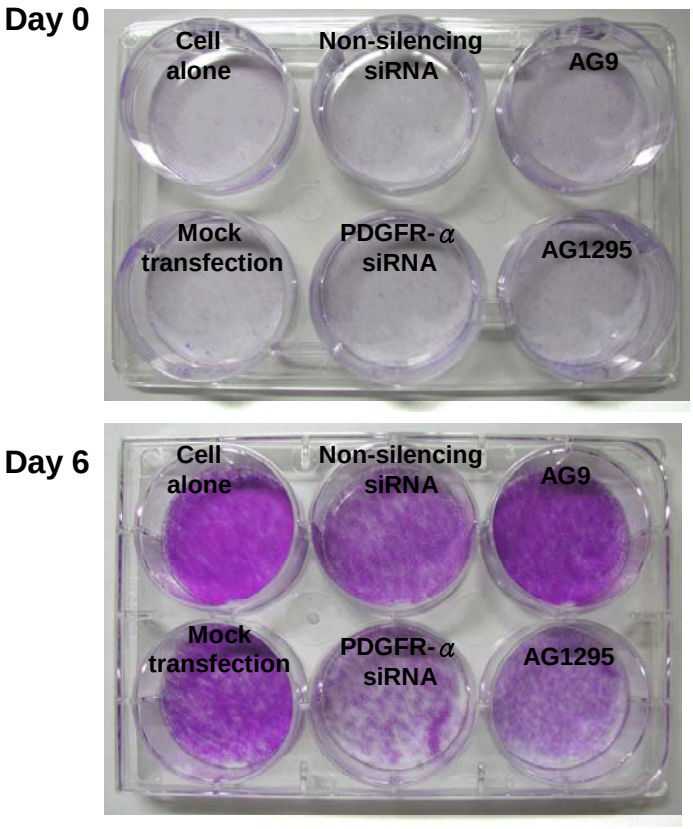


Figure 6. CGTH W-1 cell growth suppressed by PDGFR-α siRNA and tyrphostin AG1295. Cell proliferation assay: 3x 10^4 CGTH W-1 cells were seeded to each well of six-wells plate. CGTH W-1 monolayers were treated with different condition of tyrosine kinase inhibitor or genetic-specific siRNA at day 0 and day 2. The monolayers were stained with crystal violet at day 0 and day 6.