Oncogene (2005) 24, 6185–6193

www.nature.com/onc

Gene expression profiles in cells transformed by overexpression of the IGF-I receptor

Gary Loughran[1], Merei Huigsloot[1], Patrick A Kiely[1], Loraine M Smith[1], Suzanne Floyd[1], Veronica Ayllon[1] and Rosemary O'Connor*[,1]

[1]*Cell Biology Laboratory, Department of Biochemistry, BioSciences Institute, National University of Ireland, Cork, Ireland*

To identify genes associated with insulin-like growth factor-I receptor (IGF-IR)-mediated cellular transformation, we isolated genes that are differentially expressed in R^- cells (derived from the IGF-IR knockout mouse) and R^+ cells (R^- cells that overexpress the IGF-IR). From these, 45 genes of known function were expressed at higher levels in R^+ cells and 22 were expressed at higher levels in R^- cells. Differential expression was confirmed by Northern blot analysis of R^+ and R^- cells. Genes expressed more abundantly in R^+ cells are associated with (1) tumour growth and metastasis including, *βigH3*, *mts1, igfbp5 protease*, and *mystique*; (2) cell division, including *cyclin A1* and *cdk1*; (3) signal transduction, including *pkcδbp* and *lmw-ptp;* and (4) metabolism including *ATPase H^+ transporter* and *ferritin*. In MCF-7 cells IGF-I induced expression of two genes, *lasp-1* and *mystique*, which could contribute to metastasis. Lasp-1 expression required activity of the PI3-kinase signalling pathway. Mystique was highly expressed in metastatic but not in androgen-dependent prostate cancer cell lines and Mystique overexpression in MCF-7 cells promoted cell migration and invasion. We conclude that genes identified in this screen may mediate IGF-IR function in cancer progression.
Oncogene (2005) **24,** 6185–6193. doi:10.1038/sj.onc.1208772; published online 6 June 2005

Keywords: IGF-I receptor; cancer; transformation; apoptosis; Mystique; Lasp-1

Introduction

Insulin-like growth factors I and II (IGF-I and -II) exert their effects by binding to the insulin-like growth factor-I receptor (IGF-IR), which mediates cell proliferation (Flier *et al*., 1986), suppression of apoptosis (Rodriguez-Tarduchy *et al*., 1992; Harrington *et al*., 1994; O'Connor *et al*., 1997), and differentiation (Valentinis *et al*., 1999). All of these IGF-IR functions are critical for normal development and homeostasis, but the IGF-IR and its ligands also contribute to cancer by promoting tumour growth, invasion, and metastasis. In prostate cancers, IGF-IR expression is stronger in both primary and metastatic prostate cancer cells than in normal tissue (Hellawell *et al*., 2002). In a mouse model of pancreatic cancer employing targeted expression of SV40 large T antigen enforced expression of the IGF-IR resulted in development of highly invasive and metastatic tumours (Lopez and Hanahan, 2002). All these studies suggest that signals from the IGF-IR contribute to the development and progression of cancer. Consequently, the IGF-IR and its signalling pathways may be important therapeutic targets in cancer (reviewed in LeRoith and Helman, 2004).

In cell models overexpression or constitutive activation of the IGF-IR can lead to ligand-dependent growth in serum-free medium and to cellular transformation (Kaleko *et al*., 1990; Sell *et al*., 1994). Embryonic fibroblasts derived from mice homozygous for a targeted deletion of the IGF-IR (R^- cells) are resistant to transformation by certain cellular and viral oncogenes that readily transform fibroblasts derived from wild-type litter mates (Sell *et al*., 1994). The dependence of transformed cells on IGF-IR signalling is also suggested by observations that clonogenic growth, metastasis, and invasion could be reversed in tumour cell lines by inhibiting IGF-IR expression or function (Burfeind *et al*., 1996; Lee *et al*., 1996; Dunn *et al*., 1998; Brodt *et al*., 2000).

IGF-I regulates expression of genes associated with cancer progression, including β-catenin (Playford *et al*., 2000), E-cadherin (Surmacz *et al*., 1998; Mauro *et al*., 2001), and the angiogenic factor VEGF (Miele *et al*., 2000). However, the overall spectrum of IGF-IR-regulated genes that act to promote transformation or facilitate tumour progression is not yet known.

We hypothesized that critical IGF-IR-regulated genes are likely to be more highly expressed in R^+ cells, which are transformed by overexpression of the IGF-IR, than in R^- cells, which are resistant to transformation (Sell *et al*., 1994). To test this hypothesis, we used these two cell lines as a model system in which to identify IGF-IR-regulated genes that could contribute to the transformed phenotype. Gene expression profiles were analysed using suppression subtractive hybridization (SSH) in cultures where both R^+ and R^- cell lines were proliferating in the presence of FBS. Genes expressed at higher levels in

*Correspondence: R O'Connor; E-mail: r.oconnor@ucc.ie
Received 24 February 2005; revised 14 April 2005; accepted 20 April 2005; published online 6 June 2005

either cells were identified. Many of these genes are responsive to IGF-I and are expressed in tumour cells. We conclude that these genes may be mediators of IGF-IR function in cancer progression.

Results

Gapdh and IGF-IR were efficiently subtracted in R^+ and R^- cDNA

R^+ and R^- cells are likely to express many identical genes, so SSH was used to eliminate common genes by allowing cDNA generated from R^+ and R^- cells to hybridize with each other. A population of subtracted cDNAs representing an R^+ cell-enriched population was isolated. To reduce the representation of genes associated with basal cell growth and genes induced in response to short-term stimulation with IGF-I, RNA was isolated from R^+ and R^- cells proliferating in medium supplemented with FBS. The subtraction efficiency of the SSH procedure was assessed by measuring the relative abundance of *gapdh* RNA by PCR before and after subtraction (Figure 1). Amplification of *gapdh* in the unsubtracted sample occurred after cycle 23, whereas amplification of *gapdh* in the subtracted sample occurred after cycle 33. This result indicates efficient subtraction of *gapdh* from the R^+ cell-enriched cDNA pool.

R^+ cells are R^- cells that have been transfected to overexpress the IGF-IR. Therefore, subtracting R^- cDNA from R^+ cDNA would result in a subtracted cDNA population in which the IGF-IR cDNA is overrepresented. To counteract this, we spiked the R^- cDNA pool with 1 μg of IGF-IR cDNA before subtraction. PCR analysis showed that the IGF-IR had been effectively subtracted from the final R^+ cell-enriched cDNA population (Figure 1).

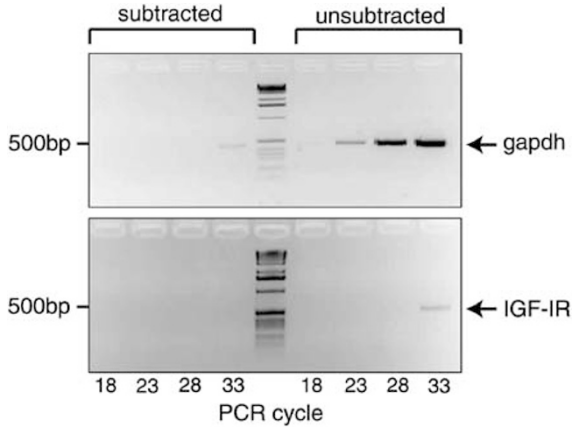

Figure 1 Evaluation of subtraction efficiency in SSH procedure. PCR was performed using primers for *gaphh* on subtracted (upper panel; left) or unsubtracted (upper panel; right) cDNA populations. Aliquots were removed during the annealing step of cycles 18, 23, 28, and 33, and fractionated on a 2.0% TAE agarose gel. Lower panel: PCR was performed with a primer set for the *igf-1r*. R^- cDNA had been spiked with 1μg of *igf-Ir* cDNA to subtract the *igf-Ir* from the R^+ population

Having confirmed the efficiency of subtraction in the SSH procedure, an R^+ cell-enriched cDNA library was then generated by cloning the subtracted cDNA population. From this cDNA library 864 randomly selected clones were amplified, purified, and then arrayed onto nylon membranes.

Differential expression of genes in R^+ and R^- cells

To identify genes that are differentially expressed in R^+ and R^- cells, subtracted probes representing either R^+ cell-enriched cDNAs or R^- cell-enriched cDNAs were hybridized to a pair of DNA arrays. These probes hybridized to approximately 50% of all the immobilized cDNAs, and 82 of them consistently hybridized exclusively, or more strongly with the R^+ subtracted probe, while 23 hybridized more strongly with the R^- probe. These clones were recovered from the cDNA library, amplified, and sequenced. There was considerable redundancy within the library because the 82 R^+ enriched clones represented 60 individual genes and the 23 R^- enriched clones represented 22 individual genes. BLAST searches of each sequence revealed that 45 clones represent previously characterized genes (shown in Table 1), which could be clustered into four functional groups (1–4). All 22 R^--enriched genes are listed in Table 2.

Group 1 contains genes that have previously been associated with tumours; these include *βig-H3*, *pebp2*, *timp3,* and *mts1* (Table 1). Group 2 contains genes associated with cell cycle or DNA replication. Group 3 contains genes associated with signalling and transcription. Group 4 contains genes associated with metabolism or ribosomal function.

The arrays were also probed with unsubtracted probes derived from R^+ and R^- cells. This resulted in detection of a much lower number of differentially expressed genes (not shown) than with the subtracted probes. This indicates that most of the differentially expressed genes (Tables 1 and 2) are expressed as relatively rare mRNAs in R^+ and R^- cells.

Genes encoding potential regulators of IGF-IR function are expressed in R^+ cells

To investigate whether the pattern of gene expression in the DNA arrays accurately reflected gene expression in R^+ and R^- cells, a subset of the identified genes were analysed by Northern blotting (asterisked in Table 1). Northern blots prepared from R^+ and R^- cell RNA were successively probed with cDNAs from the relevant clones. Densitometric analysis of the bands was used to measure the fold difference in the expression of each gene in R^+ and R^- cells (Figure 2).

Mts1, *igfbp5 protease, βigH3,* and *β-tubulin* all hybridized more strongly to RNA from R^+ cells than to RNA from R^- cells (Figure 2). The gene with greatest differential expression was *mts1* (eight-fold higher in R^+ cells). Mts1 belongs to the S100 family of small acidic

Table 1 Genes that are more highly expressed in R^+ cells compared with R^- cells

Clone name	*Homology*	*Redundancy*	*Accession*
Group 1 – tumour associated			
A10-2, N2-2, O18-1, N1-2, B16-2	IGFBP5 protease*	5	AF179369
C4-1, J12-1	Mts1/S100A4*	2	MMTS1
F3-1, O20-1, 13-2, B3-2	Nm23/ndkB*	4	NM_008705
H16-1, G16-1	Pebp2*	2	D14570
113-3	Tubulin beta*	1	X60785
J5-1	*β*ig-H3*	1	NM_009369
K7-1	ATPase subunit 6	1	AF093677
L11-2	Timp3	1	NM_011595
P16-2	NADH-ubiquinone oxidoreductase chain 3	1	P05506
Group 2 – cell cycle/DNA replication			
C17-3	CKS2*	1	NM_001827
D1-1	Cyclin A1*	1	MMCYCLA
H2-1	Rfc2	1	AF208499
P7-1	Thymidylate synthase	1	M13019
Group 3 – signalling/transcription			
A5-1	RacGAP1*	1	NM_012025
A23-1	Smu-1	1	BC057446
B19-1	Ryudocan core protein*	1	D89571
D6-2	Apoptosis related protein-15	1	NM_019745
E13-2	Alpha initiation factor*	1	X65948
F3-2	Lasp-1*	1	BC010840
F17-2	RAB18	1	NM_181070
G9-1, P6-1	GAB A – A receptor associated protein	2	AB041648
H7-2	PKC*δ* bp*	1	D85435
L9-2	LMW-PTP*	1	Y17345
N9-1, I9-3	Tis11D*	2	U07802
P13-1	Gtr2	1	NM_017475
Group 4 – metabolism/ribosomal function			
A23-2	Ferritin light chain	1	NM
B6-1, P4-1, B9-2, E3-3	Ferritin heavy chain*	4	NM
B20-2	Ribosomal protein L3	1	NM
D3-2	Ribosomal protein L37a	1	NM
E1-1	Aldolase 1, isoform A	1	NM
E7-2	Ribosomal protein S21	1	X79
E9-2	Solute carrier family 7	1	NM
G11-1, K21-3	Ribosomal protein S26	2	NM
H18-2	Human ATPase, H^+ transporter	1	NM
H21-2	Ribosomal protein S24	1	X60
M1-1, M15-1, J3-1, K12-1, A19-2	Ribosomal protein L35a	5	MM
M13-3	Calumenin	1	NM
N17-1, C9-3	Ribosomal protein L7	2	MR
O22-2	Cytochrome *c* oxidase, subunit 1	1	AB0
P5-1	Human 26s proteasome regulatory subunit	1	AF0
P20-2	Ribosomal protein L10a	1	NM
A1-2	Splicing factor 3b, subunit 5	1	BC0
A5-2	Serine carboxypeptidase 1	1	BC0
F21-2	RBM3	1	AY0
M11-3	Kif-20a	1	NM

Four sets of DNA arrays derived from R^+ subtracted cDNA library were probed with forward (R^+) and reverse-subtracted (R^-) probes that were used in the SSH. The data represent the identity of genes that hybridized more strongly to the forward probe compared to the reverse probe in at least three out of four hybridization experiments. The inserts of the clones were sequenced and identified by comparison to the DNA databases using BLAST analysis. The clone name refers to the identity of the arrayed DNA fragment and cDNA library clone from which the gene fragment was derived. Asterisks indicate genes that were selected for further analysis by Northern blotting

calcium-binding proteins associated with metastasis (Zimmer *et al*., 1995; Schafer and Heizmann, 1996). The second most abundant gene was *igfbp5 protease* (5.3-fold higher in R^+ cells), which encodes a protease for the IGF-binding protein 5 (IGFBP5) (Clemmons *et al*., 1992). The third most abundant gene was *βigH3* (four-fold higher in R^+ cells), which is a TGF-*β* regulated gene (Skonier *et al*., 1992).

It has previously been shown that the cell cycle in R^- cells is longer at every stage than in R^+ cells (Sell *et al*., 1994). Thus, it was not surprising that genes associated with cell cycle progression were more abundant in R^+ cells (Table 1). *Cyclin A1* and *cdk1/cdc2* were both more than twice as high in R^+ cells than in R^- cells (Figure 2).

Group 3 genes (Table 1) are involved in cell signalling and transcription, but many of them are also associated

Table 2 Genes that are more highly expressed in R^- cells compared with R^+ cells

Clone name	*Homology*	*Redundancy*	*Accession*
A3-3	Dp 1	1	NP_033387
A18-1	Annexin AIII	1	NM_013470
A20-2	Mystique (PDLIM2)	1	BC024556
A22-1	Filamin	1	AF119149
C7-2	Nd1-L	1	AB055737
C11-1	Ribosomal protein L9	1	AF260271
D6-1	Ube2v2	1	NM_023585
D10-2	Ribosomal protein L24	1	NP_000977
E4-1	IL-6	1	MMIFR2
E5-3	Stearoyl-CoA desaturase	1	NM_009128
E12-1	Rat CaBP1	1	Q63081
E16-2	Centrin	1	AF080592
G7-2	RB-BP7	1	AF090306
G10-1	Cytochrome *c* oxidase, subunit 1	1	AB042524
G16-2	NADH-ubiquinone oxidoreductase chain 4	1	QXMS4M
H13-2	Cdk1/cdc2*	1	AAA37408
I7-2	Thioredoxin	1	NM_011660
J18-2	PKA regulatory subunit	1	NP_03731
L17-2, P24-2	RAB1A	2	AF226873
M7-2	Human 26s proteasome regulatory subunit	1	AF006305
M21-2	Cap-g*	1	XM_132039

DNA arrays were probed as described for Table 1. The data represent the identity of genes that hybridized more strongly to the reverse probe compared to the forward probe in at least three out of four hybridization experiments. The inserts of the clones were sequenced and identified by comparison to the DNA databases using BLAST analysis. The clone name refers to the identity of the arrayed DNA fragment and cDNA library clone from which the gene fragment was derived. Asterisks indicate genes that were selected for further analysis by Northern blotting

with cellular transformation. Five of these genes were analysed by Northern blotting. The greatest difference in expression (five-fold higher in R^+ cells) was observed with PKCδ-binding protein (Izumi *et al*., 1997) (Figure 3). Low Molecular Weight-Protein Tyrosine Phosphatase (*Lmw-ptp*), which can negatively regulate growth (Fiaschi *et al*., 2001), was expressed almost four-fold more in R^+ cells (Figure 3). The early response zinc-finger protein *tis11D* (Varnum *et al*., 1991) was three-fold more abundant in R^+ cells than in R^- cells (Figure 3). The *lasp-1* gene, which encodes a member of a LIM domain family (Tomasetto *et al*., 1995a), was increased by almost two-fold in R^+ cells.

These data indicate that several genes encoding proteins that could mediate or regulate IGF-IR function in transformed cells are more highly expressed in R^+ cells than in R^- cells.

IGF-I induces expression of both R^+ cell enriched and R^- cell enriched genes

Next, we investigated whether some of the genes enriched in R^+ or R^- cells were responsive to IGF-I. The 22 known genes that were more abundantly expressed in R^- cells (Table 2) fall into broad functional categories as the R^+ cell-enriched genes do. Since the DNA arrays consisted of cDNA fragments from the R^+-enriched cDNA pool, we did not anticipate that so many genes would hybridize preferentially to the R^- cell-derived probes. One explanation may be that these genes are dynamically regulated by IGF-I and may be expressed at lower levels in R^+ cells than in R^- cells at certain times after IGF-I stimulation.

Northern blots were prepared with RNA from R^+ cells that had been stimulated with IGF-I (Figure 4). Out of the tumour-associated gene group, *pebp2* and *nm23* were induced within 2 h of IGF-I stimulation, whereas expression of *βig-H3* RNA was induced after 6 h. In contrast, *mts1* and *lmw-ptp* were equally abundant in the starved and IGF-I-stimulated cells. C*ks2* and *cdk1* showed no detectable change in the expression of RNA in response to IGF-I. C*dk1* is a gene known to be IGF-I responsive (Surmacz *et al*., 1992), so this result may be due to the high basal levels of *cdk1* in R^+ cells. The signalling and transcription-associated genes *tis11D* and *lasp-1* were induced after 2 h of IGF-I stimulation.

Next, we analysed IGF-I-induced expression of *rab1a, dp-1,* and *mystique*, all of which initially hybridized preferentially to the R^- cell probe in the DNA arrays (Table 2). The Rab1A (Pind *et al*., 1994) and Dp-1 (Bandara *et al*., 1994) proteins are associated with membrane trafficking and cell cycle regulation, respectively. Each of these genes was induced in response to IGF-I stimulation (Figure 4). *Mystique* encodes a cytoskeletal associated PDZ-LIM protein (Loughran *et al*., 2005). It was expressed as alternatively spliced transcripts (Figure 4). Altogether, the data indicate that many of the genes enriched in R^+ and R^- cells are dynamically regulated by IGF-I stimulation.

IGF-I-induced lasp-1 *expression requires PI3-Kinase activity*

To extend the observations in the R^+/R^- cell model we turned next to human tumour cell lines and investigated the products of two genes that could potentially

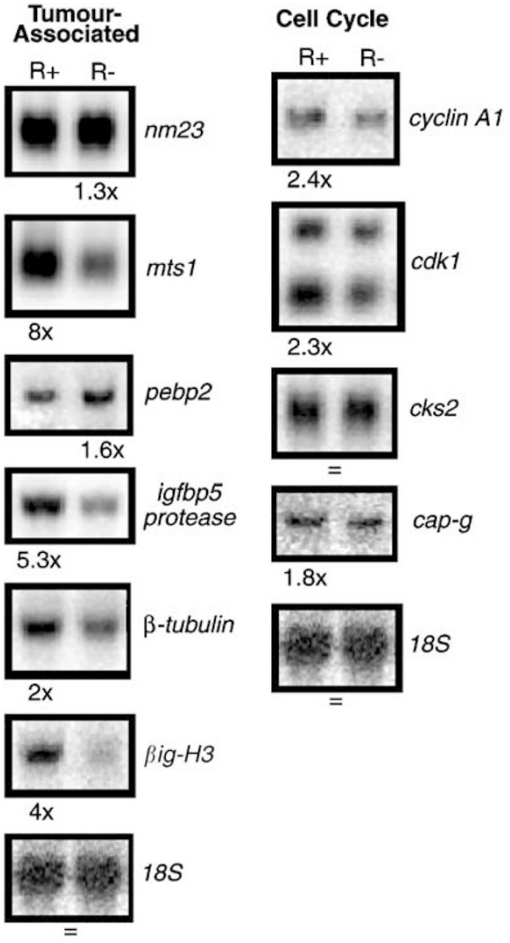

Figure 2 Expression of tumour- and cell cycle-associated genes. Northern blots were prepared with total RNA (20 μg per lane) and subjected to successive rounds of probing and stripping. Probes representing each gene (as indicated) from groups 1 and 2 in Table 1 were generated by random priming of the cDNA. Membranes were exposed to phosphorimager screens for 6–48 h and then subjected to densitometric analysis with normalization for the 18S probe. The fold-increased expression of each gene in either R^+ or R^- cells is indicated under the panels

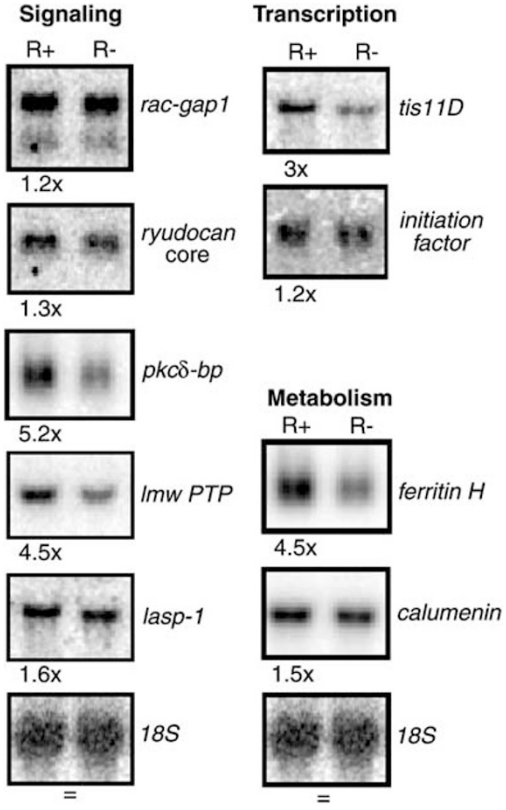

Figure 3 Expression of genes associated with signalling and transcription. Northern blots were prepared with total RNA (20 μg per lane) and subjected to successive rounds of probing and stripping. Probes (as indicated) from groups 3 and 4 in Table 1 were generated by random priming of cDNA. Blots were stripped and rehybridized, exposed to phosphorimager screens, and analysed by densitometry

contribute to cancer progression, namely *lasp-1* and *mystique*. Lasp-1 is a scaffolding protein associated with human breast cancer-derived metastases that has a LIM domain, two actin-binding domains, and an SH3 domain (Tomasetto *et al*., 1995a). First, we investigated whether human *lasp-1* was IGF-I inducible in the breast carcinoma cell line MCF-7. Lasp-1 mRNA expression was induced by IGF-I in MCF-7 cells (Figure 5a), which confirms the observation in R^+ cells (Figure 4). To determine which IGF-I-activated signalling pathways regulate *lasp-1* expression, serum-starved MCF-7 cells were pretreated with inhibitors of the PI3-kinase, mTOR and ERK pathways (Figure 5b). This indicated that IGF-I-induced *lasp-1* expression requires the PI3-K and to a lesser extent the mTOR pathway, but does not require the Erk pathway. Taken together, these data indicate that the breast cancer-associated gene *lasp-1* is IGF-I-responsive.

Mystique expression in tumour cell lines and effects on cell motility and invasion

Next, we investigated the contribution of the Mystique PDZ-LIM protein to IGF-IR function in tumour cell lines. Although the *mystique* mRNA is alternatively spliced we could only detect one protein species that was expressed more abundantly in R^+ cells than R^- cells and also expressed in MCF-7 cells (Figure 6a; Loughran *et al*., 2005). To investigate the contribution to cell migration and invasion, we overexpressed Ha-tagged Mystique in MCF-7 cells. The motility of these cells was enhanced compared to MCF-7/Neo cells and they also acquired the ability to migrate through the extracellular matrix material matrigel (Figure 6b).

Endogenous Mystique expression was high in two metastatic prostate cancer cell lines (DΔu145 and PC3) but was not detectable in two nonmetastatic cell lines (Alva-31 and PPC1). This, combined with our published observation that silencing Mystique expression with siRNA suppresses cell migration in MCF-7 and MCF10A cells (Loughran *et al*., 2005), indicates that Mystique is an IGF-I-responsive protein that could contribute to metastasis.

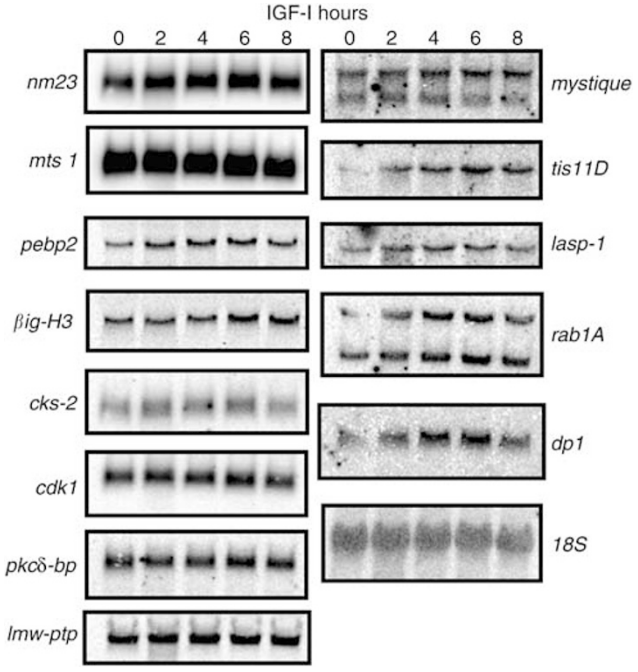

Figure 4 IGF-I induces expression of genes in R^+ cells. R^+ cells were starved for 4 h from serum and then stimulated with IGF-I (100 ng/ml) in serum-free medium for the indicated times at which times RNA was isolated. Northern blots were prepared with total RNA (20 μg) and probed as described in Figure 3

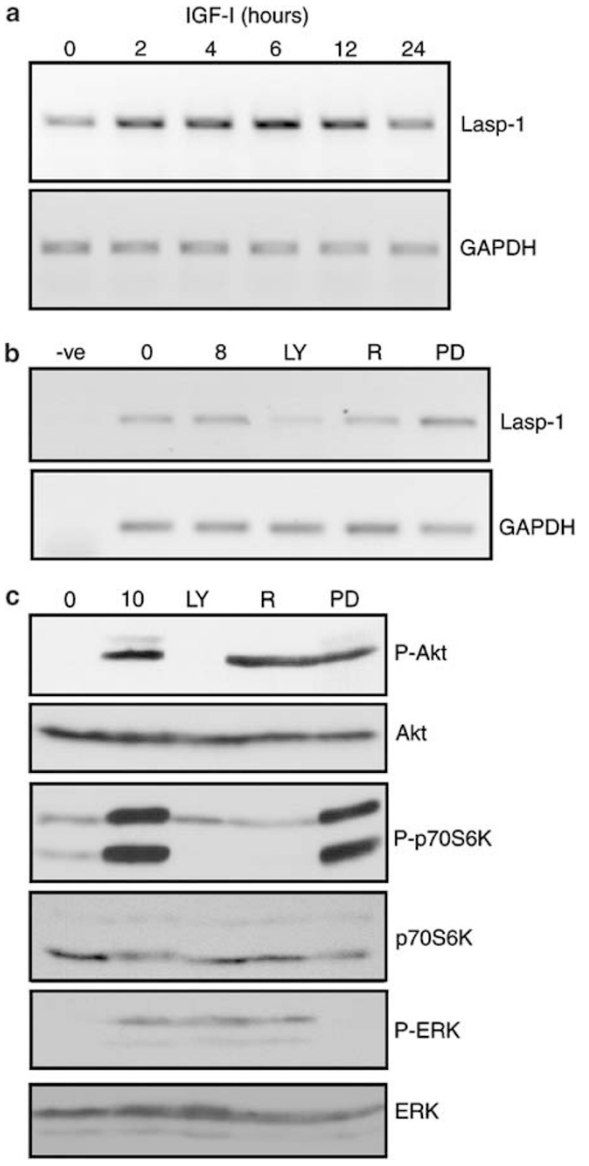

Figure 5 IGF-I-induced expression of Lasp-1 requires PI3-kinase and mTOR activity. MCF-7 cells were serum-starved overnight and stimulated with IGF-I (100 ng/ml). (**a**, **b**) Semiquantitative RT–PCR was performed for Lasp-1 with *gapdh* as loading control. (**b**, **c**) Cells were pretreated with inhibitors of PI3-K (LY294002), MEK (PD98059), and mTOR (Rapamycin) for 30 min before IGF-I stimulation for 0 or 8 h. Activity of the inhibitors was confirmed after 10 min IGF-I stimulation by Western blotting using phospho-S^{473} Akt, phospho-$T^{202/204}$ ERK1/2, and phospho-T^{389} p70S6 kinase antibodies. Akt, P70S6k, and Erk expression levels were determined as controls

Discussion

This study revealed that a wide range of genes are upregulated in R^+ cells compared to R^- cells. These genes may be important in mediating or regulating IGF-IR actions in tumours. Our study also confirms observations from previous studies indicating that the IGF-IR is broadly active in regulating the cell cycle, cell survival, motility, attachment, and metastasis (Surmacz *et al*., 1998; Miele *et al*., 2000; Mauro *et al*., 2001; Lopez and Hanahan, 2002).

The genes we identified as more abundant in R^+ cells are quite distinct from IGF-I-responsive genes in three other microarray studies. Two of these studies identified genes that are differentially expressed in response to either insulin or IGF-I stimulation (Dupont *et al*., 2001; Mulligan *et al*., 2002). The other study investigated IGF-I-induced gene expression in cardiomyocytes (Liu *et al*., 2000). One gene identified in the insulin/IGF-I studies, *nm23* (Mulligan *et al*., 2002), was also upregulated in R^+ cells in our screen. From the cardiomyocyte study, *timp3* was the only gene that was also found to be upregulated in R^+ cells. These differences may be due to the different tissue sources of the cDNA or due to the fact that our cDNA array was enriched for genes whose expression was higher in R^+ cells than R^- cells. SSH is known to enrich for RNA transcripts that are rare in cells.

Many of the genes that were differentially expressed in R^+ and R^- cells, including those encoding *tis11D*, *lasp-1*, *dp-1*, *βig-H3*, *pebp2*, *rab1A,* and *mystique*, were induced by short-term IGF-I stimulation of cells. This suggests that the protein products of these genes may be active in mediating or regulating signals from the IGF-IR. Other genes that were more abundant in R^+ cells, but were not seen to be induced by IGF-I, may be more highly expressed in R^+ cells as a result of prolonged IGF-IR-mediated signalling or as a consequence of the cells becoming transformed.

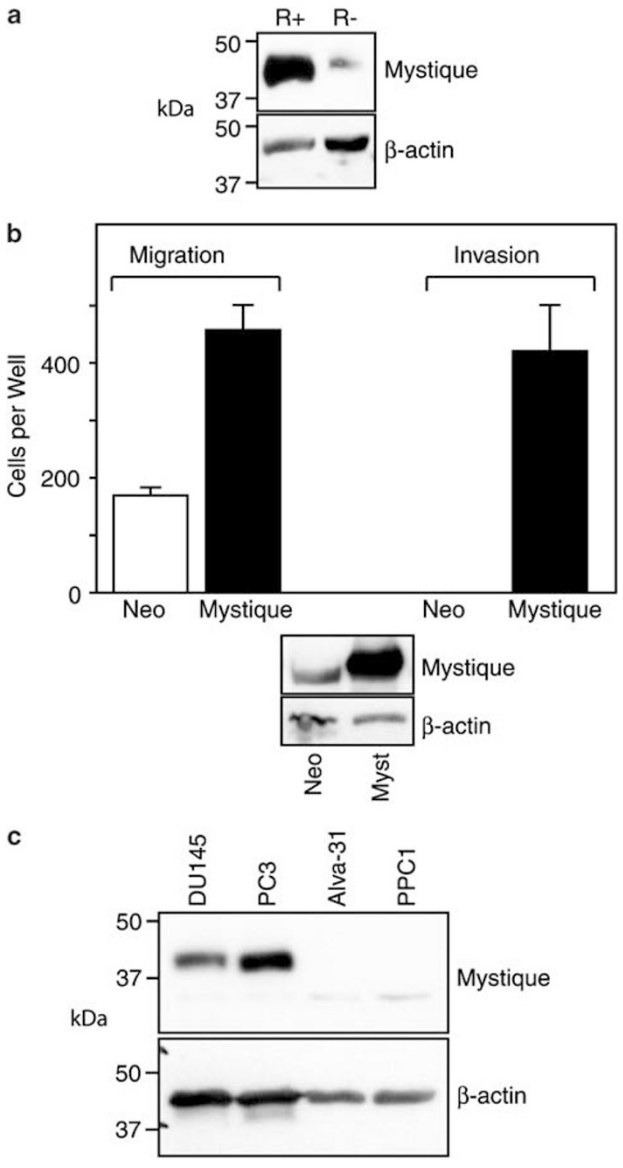

Figure 6 Mystique is expressed in tumour cell lines and can promote migration and invasion. (**a**) Western blots prepared from R^+ and R^- cell lysates were probed with the antiMystique antiserum. (**b**) MCF-7 cells overexpressing Ha-Mystique or Vector (Neo) were assessed for migration and Matrigel invasion in transwell assays. Data are presented as average of counts from triplicate wells for each test condition. Below the graph is a Western blot showing overexpression of Ha-Mystique. (**c**) Western blots prepared from the indicated prostate cell lines were probed with anti-Mystique antiserum

Several of the isolated genes (*igfbp5 protease, lmw-ptp, timp3, mts1,* and *βigH3*) encode proteins that have previously been associated with tumour cell growth, and several have a putative role in cell migration or metastasis (*timp3, mts1*, *βig-H3,* and *lasp-1*). *Mts1* was first isolated from a differential cDNA screen of metastatic and nonmetastatic tumour cell lines (Ebralidze *et al*., 1989; Ambartsumian *et al*., 1996). *BigH3* was more highly expressed in tumourigenic cells than in nontumourigenic cells (Tsujimoto *et al*., 1999).

The IGF-IR has been linked with metastatic potential (Long *et al*., 1995, 1998; Dunn *et al*., 1998; Brodt *et al*., 2000, 2001; Hellawell *et al*., 2002; Lopez and Hanahan, 2002). Our study has identified potential new mediators of this activity. These include Lasp-1 and Mystique, whose expression and function we investigated in tumour cell lines. Lasp-1 was originally found to be overexpressed in 8–12% of human breast cancer-derived metastases (Tomasetto *et al*., 1995a, b). Expression of *lasp-1* was induced by IGF-I in both R^+ and MCF-7 cells in a PI3-K pathway-dependent manner, which suggests it is a mediator of IGF-IR actions in tumours. This suggestion is supported by a recent study demonstrating that Lasp-1 is a target of the c-Abl kinase and can promote migration or cell survival by modulating cytoskeletal function through its LIM, SH3, and actin-binding domains (Lin *et al*., 2004).

The *Mystique* gene is expressed as at least three splice variants and is located on chromosome 8p21.2, which is a region frequently disrupted in cancer. We previously found that one isoform of Mystique is highly expressed in breast epithelial MCF10A cells, and its expression is regulated by IGF-I and adhesion. Silencing of Mystique protein expression suppresses cell motility and attachment (Loughran *et al*., 2005). Here we have shown that overexpression of Mystique promotes MCF-7 cell migration and invasion and that it is highly expressed in two metastatic prostate cell lines but not in androgen-dependent prostate cancer cells. This suggests deregulation of Mystique expression in metastatic cancer and indicates a role for this protein in promoting metastasis.

In conclusion, our data demonstrate that genes associated with cell growth, metastasis, and cancer progression accumulate in cells transformed by overexpression of the IGF-IR or are induced by IGF-I in these cells. Further study of these proteins may reveal new mechanisms of IGF-IR function, provide potential novel targets for therapeutic intervention, or these proteins may be useful as biomarkers to monitor the activity of the IGF-IR signalling pathway in response to drugs that target it.

Materials and methods

Cell culture, cloning, and transfection

R^- cells are mouse embryo fibroblasts that were established from mice with a targeted disruption of the IGF-IR (Sell *et al*., 1993), and R^+ cells are R^- cells that were transfected to overexpress the IGF-IR (Sell *et al*., 1994). All cell lines were maintained in DMEM (Biowhittaker UK, Berkshire, UK) supplemented with 10% FBS (complete medium). To generate cDNA for SSH and subtracted probes, RNA was extracted from cells that were cultured to approximately 70% confluence in complete medium. To measure IGF-I-induced gene expression, RNA was extracted from cells that were starved from FBS for 4 h in serum-free medium before the addition of IGF-I (100 ng/ml) for up to 8 h.

Cloning and expression of the Mystique cDNA is described in Loughran *et al*. (2005). Stable clones of MCF-7 cells overexpressing Ha-Mystique were maintained in complete medium supplemented with geneticin (1 mg/ml).

Suppression subtractive hybridization

Suppression subtractive hybridization (SSH) (Diatchenko *et al*., 1996) was performed using the PCR-select cDNA Subtraction Kit (Clontech, Basingstoke, UK) on 1 μg of mRNA, which was isolated from R^+ and R^- cells (from 5×10^6 cells) using the FastTrack 2.0 Kit (Invitrogen, Breda, The Netherlands). Synthesis of cDNA, attachment of linkers for subtraction, subtraction hybridization, and suppression PCR were all carried out following the manufacturer's instructions using the controls provided. The recovered R^+ cell-enriched PCR products were used to generate a cDNA library by ligation into the pT-Adv vector (Clontech) and transformation of the resultant clones into XL-GOLD competent cells (Stratagene, Amsterdam, Netherlands).

DNA arrays and differential screening

Inserts from 864 randomly selected clones were amplified by colony PCR using flanking primers within the pT-Adv vector. Each insert was purified using Multiscreen 96-well filter plates (Millipore, Watford, UK) and robotically arrayed in duplicate onto multiple nylon membranes.

Identical membranes were probed with forward- or reverse-subtracted cDNA probes. The forward-subtracted probes were made from the same cDNA used to construct the subtracted library. The reverse-subtracted probes were derived by subtraction in the opposite direction (R^+ cDNA from R^- cDNA). The probes were labelled by random hexamer priming (Clontech) with ^{33}P-dCTP (Amersham, Buckinghamshire, UK), and prehybridization and hybridizations were performed at 72°C using the reagents provided. Filters were washed at 68°C for 4×20 min in low stringency buffer ($2 \times$ SSC and 0.5% SDS) and 2×20 min in high stringency buffer ($0.2 \times$ SSC and 0.5% SDS) before exposure to a phosphorimager screen for 48 h (Storm 860 Molecular Dynamics Sunnyvale, CA, USA), and analysed with Image Quant and Arrayvision™ software (Imaging Research Inc., Ontario, Canada). In these arrays, cDNAs derived from mRNAs that are truly upregulated in R^+ cells are expected to hybridize preferentially to the forward-subtracted probe; therefore, clones that hybridized equally to the reverse-subtracted probes are considered as background. To control for variation within the PCR amplification, hybridization, or cDNA immobilization procedures, the differential screen was repeated four times using freshly amplified and labelled subtracted probes on newly prepared DNA arrays.

Northern blot analysis

Northern blots were performed as previously described (Loughran *et al*., 2005). Signal intensities from phosphorimages were quantified using Arrayvision™ software and corrected for differences in loading by dividing the signal intensity for each probe by the signal intensity for 18S ribosomal RNA.

Semiquantitative RT–PCR

Total RNA from 2×10^6 cells was extracted using the Trizol Reagent (Gibco-BRL). To eliminate contaminating DNA, RNA was incubated for 30 min at 37°C with DNase I (Roche, East Sussex, UK) in 10 mM Tris-Cl, pH 8.3, 50 mM KCl, and 1.5 mM $MgCl_2$. Total RNA (2 μg) was converted into cDNA using a first strand cDNA synthesis kit (Roche) in a 20 μl volume. PCR was performed in 30 μl containing 1 μl cDNA, 15 pmol of each primer, 1.5 mM $MgCl_2$, 10 mM dNTPs, and 1 unit of HotStar *Taq* DNA polymerase, in *Taq* buffer (Qiagen, West Sussex, UK). The PCR conditions were as follows: 95°C, 15 min; 94°C, 30 s; 55–58°C, 30 s; 72°C, 30–45 s; for 29 cycles (21–23 for *gapdh*). Then 5 μl of each PCR was fractionated on a 1.2% agarose gel.

Western blotting

All protein samples were resolved by SDS–PAGE on 4–20% gradient gels, and then transferred to nitrocellulose membranes. Primary antibody incubations were overnight at 4°C. Secondary antibodies conjugated with horseradish peroxidase were used for detection with enhanced chemiluminescence ECL + (Amersham). Phospho-S^{473} Akt was purchased from Biosource (Nivelles, Belgium), and Akt, Erk1/2, p70S6K, phospho-$T^{202/204}$ ERK1/2 and phospho-T^{389} p70S6 kinase were from Cell Signalling (Beverly, MA, USA). The Mystique antiserum was described (Loughran *et al*., 2005). LY294002 (20 μM), PD98059 (20 μM), and Rapamycin (20 nM) were all from CalbioChem (Nottingham, UK).

Migration and matrigel invasion assays

Migration chambers (Transwell plates Costar Ltd.) were coated on both sides with 10 μg/ml collagen I at 4°C overnight before cells were added. Invasion chambers (Transwell plates) had 100 μl ice-cold Matrigel (Stratech Scientific Ltd., Bedfordshire, UK) added to the upper surface. Lower wells were loaded with DMEM/BSA 10 ng/ml IGF-I for migration assays or DMEM/BSA 10% FBS for invasion assays. Cells (50 000) were added to upper wells. After 4 h (migration assay) or 18 h (invasion assay), chambers were disassembled and cells on the upper surface of the membrane were removed. The membrane was stained with 0.1% crystal violet and cell counts were obtained per membrane.

Acknowledgements
This work was funded by Tanaud Ireland Inc., Higher Education Authority of Ireland, Cancer Research Ireland, and Science Foundation Ireland. We are grateful to Kurt Tidmore for preparing the illustrations.

References

Ambartsumian NS, Grigorian MS, Larsen IF, Karlstrom O, Sidenius N, Rygaard J, Georgiev G and Lukanidin E. (1996). *Oncogene*, **13,** 1621–1630.

Bandara LR, Lam EW, Sorensen TS, Zamanian M, Girling R and La Thangue NB. (1994). *EMBO J.*, **13,** 3104–3114.

Brodt P, Fallavollita L, Khatib AM, Samani AA and Zhang D. (2001). *J. Biol. Chem.*, **276,** 33608–33615.

Brodt P, Samani A and Navab R. (2000). *Biochem. Pharmacol.*, **60,** 1101–1107.

Burfeind P, Chernicky CL, Rininsland F and Ilan J. (1996). *Proc. Natl. Acad. Sci. USA*, **93,** 7263–7268.

Clemmons DR, Dehoff ML, Busby WH, Bayne ML and Cascieri MA. (1992). *Endocrinology*, **131,** 890–895.

Diatchenko L, Lau YF, Campbell AP, Chenchik A, Moqadam F, Huang B, Lukyanov S, Lukyanov K, Gurskaya N, Sverdlov ED and Siebert PD. (1996). *Proc. Natl. Acad. Sci. USA*, **93,** 6025–6030.

Dunn SE, Ehrlich M, Sharp NJ, Reiss K, Solomon G, Hawkins R, Baserga R and Barrett JC. (1998). *Cancer Res.*, **58,** 3353–3361.
Dupont J, Khan J, Qu BH, Metzler P, Helman L and LeRoith D. (2001). *Endocrinology*, **142,** 4969–4975.
Ebralidze A, Tulchinsky E, Grigorian M, Afanasyeva A, Senin V, Revazova E and Lukanidin E. (1989). *Genes Dev.*, **3,** 1086–1093.
Fiaschi T, Chiarugi P, Buricchi F, Giannoni E, Taddei ML, Talini D, Cozzi G, Zecchi-Orlandini S, Raugei G and Ramponi G. (2001). *J. Biol. Chem.*, **10,** 10.
Flier JS, Usher P and Moses AC. (1986). *Proc. Natl. Acad. Sci. USA*, **83,** 664–668.
Harrington EA, Bennett MR, Fanidi A and Evan GI. (1994). *EMBO J.*, **13,** 3286–3295.
Hellawell GO, Turner GD, Davies DR, Poulsom R, Brewster SF and Macaulay VM. (2002). *Cancer Res.*, **62,** 2942–2950.
Izumi Y, Hirai S, Tamai Y, Fujise-Matsuoka A, Nishimura Y and Ohno S. (1997). *J. Biol. Chem.*, **272,** 7381–7389.
Kaleko M, Rutter WJ and Miller AD. (1990). *Mol. Cell. Biol.*, **10,** 464–473.
Lee CT, Wu S, Gabrilovich D, Chen H, Nadaf-Rahrov S, Ciernik IF and Carbone DP. (1996). *Cancer Res.*, **56,** 3038–3041.
LeRoith D and Helman L. (2004). *Cancer Cell*, **5,** 201–202.
Lin YH, Park ZY, Lin D, Brahmbhatt AA, Rio MC, Yates III JR and Klemke RL. (2004). *J. Cell Biol.*, **165,** 421–432.
Liu T-J, Lai H-C, Wu W, Chinn S and Wang P. (2000). *Circ. Res.*, **88,** 1231–1238.
Long L, Navab R and Brodt P. (1998). *Cancer Res.*, **58,** 3243–3247.
Long L, Rubin R, Baserga R and Brodt P. (1995). *Cancer Res.*, **55,** 1006–1009.
Lopez T and Hanahan D. (2002). *Cancer Cell*, **1,** 339–353.
Loughran G, Healy N, Kiely PA, Huigsloot M, Kedersha NL and O'Connor R. (2005). *Mol. Biol. Cell*, **16,** 1811–1822.
Mauro L, Bartucci M, Morelli C, Ando S and Surmacz E. (2001). *J. Biol. Chem.*, **276,** 39892–39897.
Miele C, Rochford JJ, Filippa N, Giorgetti-Peraldi S and Van Obberghen E. (2000). *J. Biol. Chem.*, **275,** 21695–21702.
Mulligan C, Rochford J, Denyer G, Stephens R, Yeo G, Freeman T, Siddle K and O'Rahilly S. (2002). *J. Biol. Chem.*, **277,** 42480–42487.
O'Connor R, Kauffmann-Zeh A, Liu Y, Lehar S, Evan GI, Baserga R and Blattler WA. (1997). *Mol. Cell. Biol.*, **17,** 427–435.
Pind SN, Nuoffer C, McCaffery JM, Plutner H, Davidson HW, Farquhar MG and Balch WE. (1994). *J. Cell Biol.*, **125,** 239–252.
Playford MP, Bicknell D, Bodmer WF and Macaulay VM. (2000). *Proc. Natl. Acad. Sci. USA*, **97,** 12103–12108.
Rodriguez-Tarduchy G, Collins MK, Garcia I and Lopez-Rivas A. (1992). *J. Immunol.*, **149,** 535–540.
Schafer BW and Heizmann CW. (1996). *Trends Biochem. Sci.*, **21,** 134–140.
Sell C, Dumenil G, Deveaud C, Miura M, Coppola D, DeAngelis T, Rubin R, Efstratiadis A and Baserga R. (1994). *Mol. Cell. Biol.*, **14,** 3604–3612.
Sell C, Rubini M, Rubin R, Liu JP, Efstratiadis A and Baserga R. (1993). *Proc. Natl. Acad. Sci. USA*, **90,** 11217–11221.
Skonier J, Neubauer M, Madisen L, Bennett K, Plowman GD and Purchio AF. (1992). *DNA Cell Biol.*, **11,** 511–522.
Surmacz E, Guvakova MA, Nolan MK, Nicosia RF and Sciacca L. (1998). *Breast Cancer Res. Treat.*, **47,** 255–267.
Surmacz E, Nugent P, Pietrzkowski Z and Baserga R. (1992). *Exp. Cell Res.*, **199,** 275–278.
Tomasetto C, Moog-Lutz C, Regnier CH, Schreiber V, Basset P and Rio MC. (1995a). *FEBS Lett.*, **373,** 245–249.
Tomasetto C, Regnier C, Moog-Lutz C, Mattei MG, Chenard MP, Lidereau R, Basset P and Rio MC. (1995b). *Genomics*, **28,** 367–376.
Tsujimoto H, Nishizuka S, Redpath JL and Stanbridge EJ. (1999). *Mol. Carcinog.*, **26,** 298–304.
Valentinis B, Romano G, Peruzzi F, Morrione A, Prisco M, Soddu S, Cristofanelli B, Sacchi A and Baserga R. (1999). *J. Biol. Chem.*, **274,** 12423–12430.
Varnum BC, Ma QF, Chi TH, Fletcher B and Herschman HR. (1991). *Mol. Cell. Biol.*, **11,** 1754–1758.
Zimmer DB, Cornwall EH, Landar A and Song W. (1995). *Brain Res. Bull.*, **37,** 417–429.