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Using Gene Expression Arrays to Elucidate Transcriptional Profiles Underlying Prolactin Function

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Prolactin is an ancient hormone, with different functions in many species. The binding of prolactin to its receptor, a member of the cytokine receptor superfamily, results in the activation of different intracellular signaling pathways, such as JAK2/STAT5, MAP kinase, and PI3K/AKT. How prolactin elicits so many different biological responses remains unclear. Recently, microarray technology has been applied to identify prolactin target genes in different systems. Here, we attempt to summarize and compare the available data. Our comparison of the genes reported to be transcriptionally regulated by prolactin indicates that there are few genes in common between the different tissues. Among the organs studied, mammary and prostate glands displayed the largest number of overlaps in putative prolactin target genes. Some of the candidates have been implicated in tumorigenesis. The relevance and validation of microarray data, as well as comparison of the results obtained by different groups, will be discussed.

KEY WORDS: prolactin target genes; expression arrays; mammary gland; prostate; ovary; uterus; Nb2 cells.

Prolactin is a peptide hormone, the bulk of which is secreted by the anterior pituitary gland. In females, prolactin serum levels are elevated during the luteal phase of the menstrual cycle (1), they increase during late pregnancy and lactation, and peak following bouts of nursing (2). It is an ancient hormone, which plays a role in very different processes in vertebrates, such as freshwater osmoregulation in fish (3), nesting behavior in birds, and growth and metamorphosis in amphibians (4). Prolactin acts pleiotropically on a variety of target tissues in mammals (5), affecting, directly and indirectly, the development of the mammary gland, the ovaries, the prostate, and lacrimal glands, the intestine, and the skin. The hormone binds to the prolactin receptor (PrlR), a member of the cytokine receptor family (6), thereby activating different intracellular signaling pathways

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Abbreviations used: 20α-HSD, 20 alpha-hydroxysteroid dehydrogenase; 3β-HSD, 3β-hydroxysteroid dehydrogenase; bHLH, basic Helix–Loop–Helix; BRCA1, breast cancer 1; C/EBP, CCAAT enhancer-binding protein; cDNA, complementary deoxyribonucleic acid; CL, corpus luteum; DNA, deoxyribonucleic acid; ECM, extracellular matrix; EGF, epidermal growth factor; EST, expressed sequence tag; GATA3, GATA binding protein 3; GlyCAM 1, glycosylation-dependent cell adhesion molecule 1; HB-EGF, heparin-binding epidermal growth factor; HE5, human epididymal protein 5; IGF-2, insulin-like growth factor 2; IRF-1, interferon-regulated factor 1; JAK, janus kinase; MAP, mitogen activated protein; MAS, MicroArray Suite; MEC, mammary epithelial cell; MIAME, minimal information on microarray experiment; mRNA, messenger ribonucleic acid; NCCR, national center of competence in research; PCR, polymerase chain reaction; PI3K, phosphoinositol 3-phosphate kinase; PPARγ, peroxisome proliferator-activated receptor γ; PR, progesterone receptor; PrlR, prolactin receptor; QRT-PCR, quantitative reverse transcriptase polymerase chain reaction; RANKL, receptor activator of nuclear factor-κB ligand; RDA, representational difference analysis; SSH, subtractive suppressive hybridization; STAT, signal transducer and activator of transcription; SVP, seminal vesicle protein; TIGR, the institute for genome research; WAP, whey acidic protein; Wt, wild type.

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such as the JAK2/Stat5 (7–9), the MAP kinase (10), and PI3K/AKT pathways (11).

In the mammary gland, prolactin signaling in the epithelium controls the formation of alveoli and the differentiation of mammary epithelial cells (MECs) into milk-secreting cells (12). Moreover, prolactin indirectly affects ductal side branching through the stimulation of progesterone secretion by the corpora lutea (13). Prolactin is also synthesized locally in the breast epithelium (14); this production may be important for cell proliferation during late pregnancy (15). Local prolactin synthesis as well as expression of the PrlR is upregulated in breast carcinomas (16), suggesting that localized deregulation of prolactin signaling may contribute to breast carcinogenesis. Consistent with this notion are observations that blocking prolactin signaling interferes with the growth of various breast cancer cell lines (17,18) and that mice lacking the prolactin gene show a delay in polyoma middle-T antigen-induced breast tumorigenesis (19). Furthermore, high serum prolactin levels have been related to an increased relative risk of developing breast cancer (20).

How can one hormone ensure milk production, control uterus, and ovaries, and affect the physiology of T cells and hair follicles?

A number of mechanisms may be responsible for these contrasting, tissue-specific effects. Different forms of prolactin have been described (21) and different receptor forms exist with distinct cytoplasmic lengths, which may differentially activate distinct intracellular signaling pathways (22). The expression of receptor and ligand isoforms in cell-type-specific constellations may contribute to the tissue-specificity of prolactin action. Alternatively, a very similar stimulus may elicit different biological responses in different target cells, the signaling networks of which are wired in distinct ways. This latter hypothesis is supported by our recent finding that a chimeric receptor consisting of the signaling domain of the erythropoietin receptor could, when coupled to the ligand binding domain of PrlR, rescue the defective alveologenesis and differentiation of $PrlR^{-/-}$ MECs (23). Recent studies exploiting new techniques for transcription profiling lend further support to this hypothesis. Our analysis of transcription profiling studies, albeit preliminary, suggest that the tissue/cell-type-specific responses at the transcriptional level differ substantially.

KNOWN TRANSCRIPTIONAL TARGETS OF PROLACTIN

In an early differential screening experiment, *interferon-regulatory factor 1 (IRF-1)* was identified as an immediate-early gene in prolactin-stimulated T cells (24). In hepatocytes, prolactin regulates transcription of the Na^+/taurocholate cotransporter (25). Furthermore, prolactin induces estrogen receptor expression (26) and progesterone secretion in the corpus luteum (CL) during pregnancy. It also represses transcription of 20-α-hydroxysteroid dehydrogenase, which plays a role in pregnancy termination in the rat (27). Another molecule known to be involved in progesterone synthesis, 3β-hydroxysteroid dehydrogenase (3β-HSD), is also regulated by prolactin in the CL (28). In NIH-3T3 cells, prolactin stimulates the transcription of the transcription factors CCAAT enhancer-binding protein (C/EBP) and peroxisome proliferator-activated receptor γ (PPARγ) (29); these effects may explain its adipogenic function in these cells.

In the mammary gland, prolactin regulates transcription of milk proteins, such as casein (30), whey acidic protein (WAP) (31) and β-lactoglobulin (32). The hormone regulates the transcription of a number of other genes in various human breast cancer cell lines. BRCA1 transcription increases in MCF-7 and T47-D cells following treatment with prolactin (33), and cyclin D1 is induced in T47D cells (34). Additional prolactin target genes were discovered using mouse MEC lines, such as the p100 coactivator in HC11 cells (35) and the CBP/p300 transactivator Cited4 in SCp2 cells (36).

DISCOVERING NOVEL MEDIATORS OF PROLACTIN ACTION THROUGH TRANSCRIPT PROFILING

Considering the variety of biological functions, the number of target genes identified is limited. How does prolactin trigger alveolar morphogenesis? What is the role of prolactin signaling in breast carcinogenesis? How does it affect other organs? One way of addressing these questions is to examine changes in gene expression induced by activation of the prolactin signaling cascade in different contexts. With the advent of microarrays, the research community has a powerful tool for surveying at once all known mRNA transcripts in a broad, unbiased fashion. Several

studies have now been published using arrays, some of them with more limited transcript sets, to learn more about genes transcribed following prolactin action in different biological processes.

PROSTATE

Using a mouse model in which prolactin is overexpressed from the metallothionine promoter, Dillner and colleagues (37) looked for genes that may be important for the prostate hyperplasias seen in these transgenic animals. cDNA from dorsolateral and ventral lobes of 4 transgenic and 5 control mice, at 4–6 months of age, was compared. Representational difference analysis (RDA) identified 152 unique sequences, 69 of which had been annotated previously while 83 sequences were novel. These 152 sequences were printed on microarrays to which total RNA from hyperplastic and control prostate tissue was cohybridized. Thirty percent of the sequences, i.e. 48 different transcripts, were detected in the starting pool of total RNA. Thirty-one percent of these, i.e. 15, were identified as significantly modulated, 10 of them were annotated. Muscle creatine kinase, vimentin, and cytochrome *C* oxidase polypeptide III were upregulated and cytokeratin 8, aldose reductase and RIL protein downmodulated in the transgenic prostates.

Robertson *et al.* (38) studied the role of prolactin in the growth of the normal and cancerous prostate of $PrlR^{-/-}$ mice by transcript profiling the dorsal and ventral lobes of prostates from 50-week-old animals. RNA was collected from five to eight lobes for each homozygous mutant and wild-type (wt) mouse. Morphological analysis of the prostates from these animals demonstrated that prolactin plays a subtle role in the morphogenesis of the prostate, since $PrlR^{-/-}$ animals displayed a 20% increase in prostate weight and a reduced epithelium content, but no alteration in branching morphology. Three experiments using Affymetrix microarrays were performed on the ventral prostate. As triplicates were not substantially more informative than duplicates, only two experiments were performed on the dorsal prostate. Genes identified as differentially expressed between $PrlR^{-/-}$ and $PrlR^{+/+}$ prostates are shown in Table I.

The majority of differentially expressed genes were decreased in the $PrlR^{-/-}$ tissue, suggesting that prolactin has overall stimulatory effects on transcription in both lobes of the prostate. Surprisingly, the genes most differentially regulated were found to be involved in fertility. Two subgroups were apparent. The first involved genes encoding proteins that mediate sperm–egg interactions, such as acidic epididymal glycoprotein HE5 and zonadhesin. Acidic epididymal glycoprotein is an androgen-regulated gene secreted by the epithelium of the epididymis that is thought to be involved in sperm–oocyte plasma membrane fusion (39–41). It is found on maturing spermatozoa and shows variable surface presentation during sperm capacitation (42). Zonadhesin is a sperm-zona pellucida binding protein that confers species specificity (43). The second subgroup contains the anti-inflammatory and procoagulant seminal vesicle proteins, which play a role in copulatory plug formation and protection of sperm from the female immune response: namely SVP2/SV-IV, the semenoclotin and seminal vesicle F protein/SVP5 (44–46). The changes in the expression

Table I. Genes Differentially Expressed Between $PrlR^{-/-}$ and $PrlR^{+/+}$ Prostates (38)

Accession #	Title	Fold change
M92849	Acidic epididymal glycoprotein 1	−4.30
M88127	Adenemoatosis polyposis coli	−0.80
X04673	Adipsin	−1.80
U03419	Alpha-1 type I procollagen	−0.80
X67140	ATPase, Ca^{++} transporting	−2.60
V00727	c-Fos	2.50
X61232	Carboxypeptidase H	−0.90
AV381191	Carcinoembryogenic antigen 10	−35.00
L38422	Carcinoembryogenic antigen 10	−27.00
AI842667	Cathepsin D	−1.20
J04953	Gelsolin	−0.77
M55561	HE5 epididymal glycoprotein	−1.80
X60676	HSP47	−1.70
U69262	Matrillin-2	−0.83
AJ223361	Myosin heavy chain 2B	−7.50
M57683	PDGF receptor, alpha polyeptide	−4.20
X58251	Procollagen, type 1, alpha 2	−1.60
Z18272	Procollagen, type VI, alpha 2	−1.80
D10214	Prolactin receptor	−5.70
D10214	Prolactin receptor	−2.95
M22957	Prolactin receptor	−6.70
M60474	Protein kinase C substrate	−1.30
X15789	Retinoic acid binding protein	−1.17
X91270	Semenoclotin	−3.60
L44117	Seminal vesicle autoantigen	−121.00
M35732	Seminal vesicle protein IV	−16.00
X57139	Seminal vesicle proteinV	−7.40
AJ005559	Small proline rich protein 2A	−10.20
U19486	SVSP99	−13.00
L48989	Troponin T3, skeletal, fast	−3.00
M28729	Tubulin alpha 1	−1.70
AI465965	Zonadhesin	−4.00

of these fertility-related genes prompted the authors to examine the fertility of $PrlR^{-/-}$ males. They found overall reduced fertility, a prolonged latency to first pregnancy and a 40% probability to produce a first pregnancy compared to $PrlR^{+/+}$ animals. This study demonstrates that transcript profiling can assist in identifying phenotypes in mutant mouse strains.

OVARY

Stocco and colleagues asked the question whether prolactin and prostaglandin F2α (PGF2α), which have opposing effects on the corpus luteum, control the expression of the same genes in an antagonistic fashion (47). Pregnant rats were hypophysectomized on day 4 of pregnancy and injected with prolactin or vehicle. Both groups were sacrificed 7 days later, and RNA was isolated from CL. Out of the 1176 distinct rat genes on the cDNA microarrays, 13 were controlled by both prolactin and PGF2α in opposite fashion. The 27 genes identified as differentially regulated by prolactin are listed in Table II.

Table II. Genes Regulated by Prolactin in the Corpus Luteum (47)

Accession #	Title	Annotation
L12382	ADP-ribosylation factor 3	Genes downregulated by prolactin
	Annexin V	
X17163	c-*jun*	
	20a-HSD[a]	
U47287	PGF2 alpha-R[a]	
M20637	Phospholipase C d1[a]	
X52498	TGF beta1[a]	
	P450scc	Genes upregulated by prolactin
	3b-HSD	
	SR-B1	
	Beta2-microglobulin	
L31884	TIMP2	
X14209	Cytochrome *c* oxidase IV	
M36320	Cathepsin H	
Y00697	Cathepsin L	
	Cu/Zn SOD	
L29259	Elongation factor SIII	
	HSP60	
D10021	ATP synthase D	
M26199	LH-R[a]	
U22424	11b-HSD2[a]	
	MGST[a]	
	GST m2[a]	
D10862	Id1[a]	
D10863	Id2[a]	
D10864	Id3[a]	
M86870	Calcium binding protein 2[a]	

[a] Genes that are antagonistically regulated by prolactin and PGF2α.

Nb2 RAT LYMPHOMA CELLS

Bole-Feysot *et al.* (48) used the rat Nb2-11c lymphoma cell line, which depends on prolactin for proliferation, to identify Prl target genes. By means of mRNA differential display, RDA, subtractive suppressive hybridization (SSH), analysis of weakly expressed genes and screening of an organized library, they identified 70 genes that were differentially transcribed by Nb2-11c cells within 2–24 h of prolactin treatment. Twenty of these were unknown; the other 50 genes were classified to 10 functional categories, such as cell cycle regulators, nucleotide metabolism/DNA replication and repair proteins, cell-surface antigens/adhesion proteins, signaling moleciles involved in apoptosis or proliferation, or transcription factors (see Table III).

UTERUS

Deletion of the PrlR in mice causes female infertility and failure of implantation (49). Applying mRNA differential display to $PrlR^{-/-}$ and wt uteri implantation sites, Baran *et al.* isolated 45 transcripts, out of which 29 corresponded to known mouse ESTs (see Table IV) (50).

MAMMARY GLAND

To identify new prolactin target genes in the mammary gland, Hou *et al.* made use of mice that are homozygous for a disrupted prolactin allele ($Prl^{-/-}$) (51). $Prl^{-/-}$ females are infertile but their mammary glands develop a normal ductal system. The authors compare RNA from $Prl^{-/-}$ and $Prl^{+/-}$ mammary glands from 10-week-old females, pooled from five mice of each genotype. One hundred seventy-nine out of 588 transcripts represented on the Clontech® membrane were detected (30%), 33 (18%) were elevated in the $Prl^{+/-}$ mammary glands, and 7 (4%) were elevated in $Prl^{-/-}$ mammary glands. The authors focused on one particular gene, the expression of which was decreased in the $Prl^{-/-}$ mammary glands, the *glycosylation-dependent cell adhesion molecule 1 (GlyCAM1)*. Immunohistochemistry confirmed that GlyCAM1 protein is expressed in the mammary epithelium and the ductal lumen of $Prl^{+/-}$ virgin mice. In the mouse mammary epithelial cell line HC11, which can be induced to express milk proteins in vitro, *GlyCAM1* mRNA is increased after 48 h of prolactin and progesterone treatment. An 800 bp fragment of the

Table III. Transcripts Modulated by Prolactin in the Rat Nb2-11c Lymphoma Cell Line (48)

Title	Annotation	Title	Annotation
Cyclin E1		Myosin heavy chain	Cytoskeleton
EGR-1	Transcripts that have a	Tubulin, a, b	
Cdc5-like protein	cell-cycle modulated	Beta-actin	
Cdk2, Cdk5	homolog in yeast	FAKp125	
Cyclin B1	Cell-cycle-modulated	IRF-1	Transcription factor
Cyclin B2	transcripts with a yeast	c-Myc	
Cyclin D2	homolog (not modulated)	c-Fos	
Cyclin D3		MRG1-related protein	
Cdc21 homolog	Nucleotide metabolism,	E2F-1	
Spermidine *N*-acetyl	DNA replication and	Zfx	
Ornithine decarboxylase	repair	Cyclophilin	Heat shock, stress response
S-adenosylmethionine		TCP-1 e and h	and chaperones
Prothymosin a		Hsp70	
PARP-2		Hsp70-like = Nb29	
Prolactin receptor	Surface molecules	Hsp27	
T-cell receptor gamma chain		Hsp86	
GnRH receptor		Beta-actin	
Glucocorticoid receptor		Alpha2-tubulin	
Galectin-8		Rdnuc	
CD45		Myosin heavy chain	
Vitamin D3 receptor		Focal adhesion kinase	
Thromboxane A2		Phosphoglycerate kinase	Metabolism
Beta2-microglobulin		Enolase a	
p38 Map kinase	Signaling molecules	Aldehyde dehydrogenase	
Pim-1		ATP synthase beta subunit	
Gfi-1		L15 type	Ribosomal proteins
Stathmin		S8, S13	
P13 kinase p110a		Itm1	Glycosylation factor
Phospholipase Cy1		EF-2	Elongation factor
14-3-3 h and e		CRM-1	
Bax		Sec-22	Intercompartment
p53		Glycine transporter	transport and trafficking
RexB/NSP		FGF-responsive	Unknown function
Phosphatidylinositol		Histones H2A, H2B	Chromatin Structure
Alpha 4 phosphoprotein		FGF-2	Growth Factor

GlyCAM1 promoter, in front of a luciferase gene, gives a threefold increase of luciferase activity in the presence of prolactin. Thus, the microarray approach allowed the authors to identify a new prolactin target gene in the mammary epithelium. The biological importance of GlyCAM1 in mediating the response to prolactin remains to be evaluated.

Using organ cultures, Naylor *et al.* demonstrated that the neuropeptide galanin acts on the mammary gland to ensure final stage differentiation and milk secretion (52). Transcript profiling of explants treated with either galanin, prolactin, or both factors was performed. The genes identified as transcriptionally regulated by either factor or both are listed in Table V. Among them were milk protein genes, markers of MEC differentiation, and several known JAK/STAT target genes.

The observation that some genes were regulated exclusively by prolactin and galanin in conjunction suggests that the two hormones can act synergistically in addition to having independent actions.

The overall number of genes regulated by prolactin was larger than the number of genes regulated by galanin. This result suggests that prolactin has a much stronger effect on transcription in the mammary gland than galanin. Interestingly, prolactin and galanin have opposite effects on the transcription levels of some of the genes which are regulated by both of them, reflecting the complexity of the interactions occurring in the mammary gland organ cultures following galanin and prolactin treatments.

As discussed above, prolactin acts as a potent mitogen and morphogen on the mammary epithelium in vivo (53), but the mechanisms by which it

Table IV. Differentially Expressed Transcripts in the PrlR$^{-/-}$ Uterus at Day 5.5 of Pregnancy (50)

Accession #	Title	Annotation	Change
AK011085	HSPC 307	Immunity	+
AF039584	Decay accelerating factor precursor		−
AE000664	T cell receptor beta		−
AJ242912	Disintegrin metalloprotease		+
AJ133291	Calpain 7		−
AJ251880	MTF-1	Machinery	−
BG063008	TIS		−
U57343	Meis 2		−
X70398	Neuronal protein		−
AK014555	KIAA0056		−
C88153	Rapamycin binding protein		−
AK011778	FK506 binding protein		−
AW3211326	Retinoblastoma binding protein		+
BC002185	SCAMP1	Cell components	−
AF105268	Glypican-6		+
L49502	Phosphoprotein		−
D10837	Lysyl oxidase		−
BC005503	Protein FLJ1		−
AK018731	Ribosomal protein L24		−
AK005722	Glutathione S-transferase		−
AF242319	Endosomal protein		−
AF362729	Ion transport regulator CHIF		+
A1648789	Immunoglobulin binding protein 2	Cell cycle	+
AB030204	Integral membrane protein E25B		−
AJ130977	Ariadne protein		−
BJ797450	Sterol O-acyltransferase 1	Metabolism	−
AF017175	Carnitine palmitoyltransferase 1		
AI853806	Glutamate receptor, AMPA 1		−

induces cell proliferation remain unclear. We showed that the cell cycle protein, cyclin D1, is required in the mammary epithelium for prolactin-induced alveologenesis. While prolactin application to primary MECs induced only a low level of cyclin D1 synthesis, longer exposures—18 h—led to a significantly higher cyclin D1 protein expression (54). These observations suggested that prolactin might be inducing the expression of an intermediate growth factor, which in turn induced cyclin D1 and proliferation. To identify such a growth factor, we conducted a screen that selects specifically for genes that function downstream of the PrlR and upstream of cyclin D1 (54). We compared the transcription profiles of pairs of contralateral cleared inguinal mammary fat pads, which had been reconstituted with *PrlR*$^{-/-}$ or *cyclin D1*$^{-/-}$ MECs. The host mice were wt. At 3 weeks of age, their inguinal fat pads were surgically cleared of endogenous epithelium and engrafted with MECs from *PrlR*$^{-/-}$ and *cyclin D1*$^{-/-}$ females. As first shown by DeOme (55), such epithelial grafts can reconstitute the mammary stroma, forming a new ductal system. Four weeks later, when the grafts had fully penetrated the fat pad, the hosts were mated. Since the morphogenetic block shown by MECs of these two genotypes are similar, these two types of mammary glands, assayed at an identical day of pregnancy, are presumed to carry comparable numbers of cells in comparable states of proliferation. The only differences in gene expression pattern between them should involve those genes that lie downstream of PrlR signaling and upstream of cyclin D1 expression.

Out of the 6500 transcripts surveyed, 319 were expressed at more than threefold higher levels in the *cyclin D1*$^{-/-}$ than in the *PrlR*$^{-/-}$ grafts, whereas 430 transcripts were downregulated more than threefold. Previous observations had indicated that, in contrast to the *PrlR*$^{-/-}$ epithelium, the *cyclin D1*$^{-/-}$ epithelium retains its ability to undergo differentiation, as manifested by its ability to synthesize milk proteins late in pregnancy (56). Accordingly, many genes encoding proteins secreted with the milk or involved in milk production, such as metabolic enzymes, calcium transport genes, and intracellular vesicular trafficking genes, were differentially expressed, with their transcripts being found at far higher levels in the *cyclin D1*$^{-/-}$ grafts than in the fat pads engrafted with *PrlR*$^{-/-}$ MECs. In addition, genes involved in signal transduction and in the construction of the cytoskeleton and the extracellular matrix (ECM) were preferentially expressed in the *cyclin D1*$^{-/-}$ recombinants.

An overview of the genes whose expression levels differed by more than 10-fold is given in Table VI. We concentrated our further analysis on one of the secreted factors that were particularly highly expressed in the *cyclin D1*$^{-/-}$ grafts, namely *insulin-like growth factor-2 (IGF-2)*, which was found at a level 13.2-fold higher in the glands engrafted with *cyclin D1*$^{-/-}$ epithelia than in their *PrlR*$^{-/-}$ counterparts. QRT-PCR showed a 30-fold difference. Consistent with a model in which prolactin relies on a secreted factor IGF-2 to induce a proliferative response, we found that prolactin induces *IGF-2 mRNA* expression in primary MECs and

Table V. Genes Found to be Regulated by Prolactin in Mammary Gland Organ Cultures (52)

Accession #	Title	Annotation	Change
V00856	Whey acidic protein	Milk protein	+
M36780	Casein alpha		+
D10215	Casein gamma		+
X04490	Casein beta		+
M87863	Alpha lactalbumin		+
M10114	Casein kappa		+
V00740	Casein delta		+
X93037	WDMN1		+
M93428	Glycosylation depend. cell adhes. mol. 1	Cell adhesion	+
U03419	Procollagen, type 1, alpha 1		+
X58251	Procollagen, type 1, alpha 2		+
X60367	Cellular retinol binding protein 1	Retinol metabolism	+
AF049702	E74-like factor 5	Transcription factor	+
Y07688	Nuclear factor I/X	Transcription factor	+
X04480	Insulin-like growth factor 1	Growth factor	+
M31680	Growth hormone receptor	GH signalling	+
L12447	IGF binding protein 5	IGF signalling	+
M38337	Milk fat globule-EGF factor 8	Cell adhesion	+
X95279	Spot 14	Lipogenesis	+
M81445	Connexin 26	Gap junction	+
AF077861	Helix–Loop–Helix inhibitor protein id2	Transcription inhibitor	+
X16490	Plasminogen activator inhibitor 2	Protease inhibitor	−
M33960	Plasminogen activator inhibitor 1		−
D10837	Ras recision gene	Tumour suppressor	−
M19681	PDGF-inducible protein (JE) gene	Chemoattractant	+
D29678	Cyclin-dependent kinase 5	Cell cycle	+
L42115	Solute carrier family 1, member 7	Transport	+
U82758	Claudin 5	Tight junction	+
AI154710	Zinc finger protein 125	DNA binding	−
D50311	Myocyte enhancer factor 2B	Transcription regulator	−
M57683	PDGF receptor beta	Tyr kinase receptor	+
L19932	Beta ig-h3	Cell adhesion inhibitor	+
J02872	Granzyme G	Proteolysis	−
L32838	Interleukin 1 receptor antagonist	ILR1 signalling	+
D38046	Topoisomerase (DNA) II beta	DNA metabolism	−
X83934	Ryanodine receptor 3	Calcium homeostasis	+
AV362816	Steroidogenic acute regulatory protein	Steroid hormone biosyn	−

that IGF-2 in turn can induce expression of cyclin D1 protein. In the absence of IGF-2 from the mammary epithelium, alveologenesis is delayed. Ectopic expression of IGF-2 in *PrlR*$^{-/-}$ epithelium rescues the proliferative defect, arguing that IGF-2 is a physiologically important mediator of prolactin-induced proliferation/morphogenesis. Hovey *et al.* independently drew the same conclusion based on transcript quantification in *PrlR*$^{-/-}$ mouse mammary glands (57).

Certainly, more can be learned from the data obtained with this screen. Another growth factor found to be differentially expressed is *HB-EGF* (Gass and Brisken, unpublished observations), and its role in alveologenesis remains to be studied. In further exploring the data, we need to be aware of several potential limitations of our approach. This screen is based on the assumption that the genes expressed at higher levels in the fat pads reconstituted with *cyclin D1*$^{-/-}$ epithelium than in the fat pads reconstituted with *PrlR*$^{-/-}$ epithelium are transcriptionally activated by prolactin signaling. However, it is formally possible, that differences in gene expression levels are independent of prolactin signaling and result instead from a lack of cyclin D1-dependent repression. This concern can be addressed by comparing expression levels in *cyclin D1*$^{-/-}$ versus *cyclin D1 wt* transplants, as done for IGF-2. More general limitations, such as the choice of genes arrayed and the sensitivity, are discussed below.

We used a novel approach to elucidate the transcriptional response to prolactin specifically in the mammary gland during early pregnancy. By transcript

Table VI. Genes Expressed at a 10-Fold Higher Level in PrlR$^{-/-}$ Mammary Epithelial Transplants than Cyclin D1 Transplanted Glands (54)

Accession #	Title	Annotation
X93037	WDNM1	Milk secretion
W18308	Ferritin heavy chain gene	
X04673	Adipsin	
X61431	ACYL-COA-binding protein	
Y00516	Fructose-bisphosphate aldolase A	
M32599	Glyceraldehyde-3-phosphate dehydrogenase	
AA071776	Phosphoglucose isomerase	
J05277	Hexokinase	
X04490	Casein beta	
L09104	Glucose phosphate isomerase	
M21285	Stearoyl-Coa desaturase	
AA117004	KDEL receptor	
X02520	Lactate dehydrogenase 1, A chain	
W09506	Fatty acid-binding protein	
X97991	Calcitonin	Calcium metabolism
M27844	Parvalbumin	
W20937	Calcium-transporting atpase sarcoplasmic reticulum type	
X51438	Vimentin	Cytoskeleton
AA168865	Actin 1 (fragment)	
J04953	Gelsolin	
X14425	Profilin 1	
U20365	Muscle gamma actin	
AA002605	Insulin-like growth factor II	Growth factors
X04017	SPARC	Extracellular matrix
X14194	Nidogen	
X17069	COL1A2	
X65582	Procollagen, type VI, alpha 2	
U08020	Alpha 1 type I collagen gene	
ET61037	Lectin	
X72862	Beta-3-adrenergic receptor	
X73523	Matrix G1a protein	
W75072	Procollagen, type IX, alpha 2	
X15358	Insulin-like growth factor binding protein 4	Signal transduction
W65899	Guanine nucleotide-binding proteinbeta subunit 2	
D10024	Calpactin I heavy chain	
X58251	E-selectin ligand-1	
L23108	GTP-binding protein	
X85788	DCC tumour suppressor	
L09192	Cathepsin D	
M16358	RAB1	
AA163643	Heat shock cognate 71 KD	Heat shock
U73744	Heat shock 70	
AA105022	Heat shock protein HSP 90-beta	
W41817	C oxidase subunit VIII precursor	Miscellaneous
Z83368	RPS3a	
M76131	Elongation factor 2	
X54691	Cytochrome C oxidase, subunit IV	
W88176	Thiol-specific antioxidant protein (PRP)	
Z50159	Sui 1	
M24263	Testosterone 16-alpha-hydroxylase gene	
AA138107	COX7c1	
D10024	Apolipoprotein E	
X82067	Thioredoxin-dependent peroxide reductase	

profiling $PrlR^{-/-}$ and $PrlR^{+/+}$ epithelial transplants during early pregnancy, we reduced the effect of differences in epithelial cell number seen during late pregnancy, when $PrlR^{-/-}$ epithelium fails to form alveoli. Additionally, we examined transcript profiles of $PrlR^{+/+}$ mammary fat pads cleared of epithelium to distinguish transcripts present in the epithelium from those in the fat pad. The profiling was performed with pools of RNA from four to six animals on high-density oligonucleotide arrays (Affymetrix MGU74A GeneChips). Data were analyzed using MicroArray Suite 4.0 (MAS 4.0 Affymetrix) and sorted using Excel (Microsoft). Fold changes, calculated by MAS 4.0 for a number of genes were confirmed by quantitative PCR using the LightCycler (Roche). Overall, there was a dramatic loss of expression in the $PrlR^{-/-}$ epithelial transplants at all 3 days of pregnancy profiled (days 2, 4, and 6), including epithelial markers such as keratins. Nevertheless, merely 15% of "epithelial transcripts," as defined by their presence in $PrlR^{+/+}$ transplants and concomitant absence from $PrlR^{+/+}$ cleared fat pads, displayed decreased expression levels in the $PrlR^{-/-}$ transplants. These results suggested that the changes in gene expression were due to a lack of prolactin action and not due to differences in epithelial cell number.

The genes identified by MAS 4.0 were sorted into groups, depending on their gene ontology as annotated in NetAffx (Affymetrix). This abbreviated list (to be published in full elsewhere; Harris *et al.*, submitted) does not include cDNAs of unknown function or genes associated with expressed sequence tags (ESTs), which are the focus of ongoing investigations. Extensive literature searches suggest that many of the genes whose expression decreased in $PrlR^{-/-}$ transplants are usually upregulated during pregnancy and localize predominantly to the epithelium.

Thus, four milk protein genes (*casein* α, β, κ, and *WDNM1*) were decreased in the glands reconstituted with $PrlR^{-/-}$ epithelium. The isolation of ß-*casein*, a known prolactin-regulated gene, confirms that our model can identify prolactin-regulated genes involved in MEC differentiation.

The development of the mammary gland is not only influenced by systemic hormones but also determined by the cell's microenvironment. One component of this environment which helps to determine tissue-specific gene expression is the ECM (58). As shown in Table VII, we identified several ECM components, involved in cell adhesion and alveolar development as potential prolactin target genes, including *procollagen*α*1 type VII and XIV*, and *laminin*β3.

Table VII. Genes Reduced in Expression During Early Pregnancy in the $PrlR^{-/-}$ Mammary Epithelial Grafts (59)

Accession #	Title	Annotation
X15662	Keratin complex 2, basic, gene 8	Cytoskeleton
M36120	Keratin complex 1, acidic, gene 19	
M13805	Keratin complex 1, acidic, gene 17	
M22832	Keratin complex 1, acidic, gene 18	
U43298	Laminin, beta 3	Cell adhesion
U32107	Procollagen, type VII, alpha 1	
AJ131395	Procollagen, type XIV, alpha 1	
X55123	GATA binding protein 3	Transcription factor
X94694	Transcription factor AP-2, gamma	
AF09505	Claudin 3	Cell junction
AF087825	Claudin 7	
M81445	Connexin 26	
AF019048	RANKL	Ligand
L41352	Amphiregulin	
M89797	Wnt-4	
M36780	Casein alpha	Milk protein
X04490	Casein beta	
M10114	Casein kappa	
X93037	WDMN1	

Claudins are recently discovered integral membrane proteins that are major structural components of tight junction strands. Tight junction closure increases during lactation to prevent diffusion of molecules across the mammary epithelium. This process is mediated by progesterone withdrawal following parturition and requires activation of the PrlR (59). Our finding that *claudin 3* and *7* expression is decreased in $PrlR^{-/-}$ mammary glands suggests that prolactin may further influence these junctions by regulating the transcription of their components.

Connexin-26 is a member of a large family of proteins that form gap junctions and allow exchange of small ions between epithelial cells. *Connexin-26* mRNA and protein expression are significantly upregulated during pregnancy and remain elevated during lactation (60); expression is confined to the alveolar epithelium and the protein localizes to sites of cell contact (61).

Our screen also identified several secreted ligands of importance for cell–cell communication and cell differentiation. One such gene, *Wnt-4*, is a

member of the Wnt family of secreted glycoproteins implicated in cell–cell signaling. We have shown that Wnt-4 acts downstream of progesterone to induce ductal side-branching during pregnancy (62). Overexpression of Wnt-4 in the mammary gland by retroviral delivery resulted in increased ductal side branching and alveolar-like structures in virgin animals (63). Another gene of interest, *amphiregulin*, is a member of the epidermal growth factor (EGF) family, whose members bind to the EGF receptor family. In mice lacking both amphiregulin alleles, the ductal tree fails to fully penetrate the fat pad. Ectopic expression of amphiregulin in the mammary epithelium can result in hyperplastic ducts and lobules (64). Finally, tumor necrosis factor (ligand) superfamily member 11, also known as receptor activator of nuclear factor-κB ligand (RANKL) and osteoprotegrin ligand, was found to be decreased in the $PrlR^{-/-}$ epithelium at all three time points. The mammary glands of $RANKL^{-/-}$ mice, similar to $PrlR^{-/-}$ mammary glands, fail to develop alveoli, resulting in lactational failure in the mutant animals. Our preliminary studies have shown that prolactin modulates *RANKL* expression during early pregnancy, suggesting that prolactin is the master regulator of signaling events necessary for alveolar development.

Interestingly, all three, *Wnt-4* (62), *amphiregulin* (65), and *RANKL* (54), are known targets of progesterone signaling. It remains to be seen whether they are also direct prolactin targets or whether their expression is indirectly controlled by prolactin. Grimm *et al.* (66) demonstrated that $PrlR^{-/-}$ mammary epithelial grafts showed increased progesterone receptor expression compared to their wt counterparts and a decrease in proliferation after combined estrogen and progesterone treatments. These findings suggest that progesterone response of the mammary gland is altered in the absence of prolactin signaling. Furthermore, studies by Hovey *et al.* (79) demonstrate that the *PrlR* and *progesterone receptor* (mRNA) show similar expression patterns, and synergistic effects of both hormones arguing further for cross talk of these two signaling pathways.

Eventually, a number of transcription factors were discovered to be important for the prolactin-stimulated development of alveolar cells, among them the GATA binding protein 3, a member of a family of transcription factors that bind to DNA through a highly conserved zinc finger domain.

Thus, these transcript profiling experiments show that, in addition to the induction of milk proteins at the late stage of differentiation, prolactin also induces the transcription of genes important for intracellular and extracellular structure, cell permeability, cell–cell communication, and differentiation.

COMPARISON OF THE DIFFERENT SCREENS

The recent studies presented above have applied expression profiling techniques to investigate the influence of prolactin on transcription in different tissues or cell lines and have generated a list of putative prolactin targets. Our comparison of the genes identified indicates that few of them are common to different tissues (see Table VIII and Table IX).

As illustrated by the Venn diagram in Fig. 1, among the different organs studied, mammary and prostate glands displayed the largest overlap in putative prolactin target genes. Three of the genes shared between these two were equally modified in the ovary; these code for proteins of the cathepsin, cytochrome c oxidase, and annexin families. On the other hand, transcription of genes coding for elongation factors of the E2F family was affected in the ovary, Nb2 cells and the mammary gland, whereas phospholipase genes were differentially transcribed in the prostate, the ovary, and the lymphoma cell line Nb2. The meaning of these findings remains open for speculation.

Cathepsins are lysosomal cysteine proteases that are optimally active in the slightly acidic-reducing milieu of lysosomes and that participate in the degradation of lysosomal proteins (67). Several members of the cathepsin family, such as cathepsin B and D, were found to be involved in the processes of apoptosis, tumor invasion, or metastasis (68), and their increased expression is correlated with poor prognosis in colorectal carcinoma (69). Interestingly, cathepsin L was also reported to play an active role in mouse mammary gland involution (70). It is conceivable that cathepsins play a role in tissue remodeling in the prostate and the mammary gland, both of which are organs that undergo extensive branching morphogenesis during their hormonally controlled development.

Annexins are Ca^{2+} and phospholipid-binding proteins, members of a conserved multigene family which are expressed throughout animal and plant kingdoms. They are known to participate in the regulation of membrane organization, membrane traffic, Ca^{2+} ion currents across membranes, and intracellular Ca^{2+} concentrations. Annexin II and V were shown to play roles in heart diseases, coagulation, cell growth, and transformation. Moreover, annexins promote insulin secretion and induce secretion in neutrophils,

Table VIII. Genes Common to Two or More Genomic Screens Outlined in This Paper

Accession #	Title	Annotation	Dillner (prostate)	Robertson (prostate)	Stocco (ovary)	Bole (Nb2)	Baran (uterus)	Naylor (mammary gland)	Brisken (mammary gland)	Ormandy (mammary gland)
M81445	Connexin 26	Gap Junction						*		*
AJ223361	Myosin heavy chain 2B	Cytoskeleton		*		*				
J04953	Gelsolin			*					*	
X15662	Keratin complex 2, basic, gene 8		*							*
M28729	Tubulin alpha 1			*		*				
X51438	Vimentin		*						*	
X04490	Casein beta	Milk protein						*	*	*
X04673	Adipsin			*					*	
X93037	WDMN1							*	*	*
L09104	Glucose phosphate isomerase							*	*	
M93428	GlyCAM 1	Cell adhesion						*and Hou *et al.*		
U03419	Procollagen, type 1, alpha 1	Extracellular matrix		*				*	*	
X58251	Procollagen, type 1, alpha 2			*				*	*	
X65582	Procollagen, type VI, alpha 2			*					*	
	Beta2-microglobulin	Surface molecules			*	*				
V00727	c-Fos	Transcription factor		*		*				
AI842667	Cathepsin D	Protease		*					*	
AF077861	Inhibitor protein ID2	Transcription inhibitor			*			*		
AA002605	Insulin-like growth factor II	Growth factors							*	
M57683	PDGF receptor alpha	Tyr kinase receptor		*				*		
M20637	Phospholipase C d1	Signalling molecule			*	*				
	T-cell receptor	Immunity				*	*			
M76131	Elongation factor 2	Miscellaneous				*			*	

*Hovey *et al.*

both in presence of Ca^{2+} (71). The modulation of annexin genes in prostate and mammary gland may relate to the secretory function of both organs.

In view of prolactin's function as a morphogen and differentiation inducing factor, it is easy to rationalize that several genes involved in protein synthesis, such as elongation factor 2 and different ribosomal proteins, are upregulated by the hormone in several target tissues. The prominent induction of cytochrome *C* oxidase subunits and heat shock proteins, however, remains puzzling to us.

Id proteins (Inhibitor of differentiation or Inhibitor of DNA-binding), differentially transcribed in ovary and mammary glands, are proteins acting as dominant negative inhibitors of differentiation-specific basic Helix–Loop–Helix transcription factors. They inhibit cellular differentiation and induce proliferation by modulating different cell cycle regulators, by both direct and indirect mechanisms (72). Recent reports show that Id proteins are overexpressed in various cancers (73). The mammary glands of $Id2^{-/-}$ mice fail to form alveoli during pregnancy (74).

Several of the genes mentioned have been implied to play a role in tumorigenesis; their transcriptional modulation by prolactin is suggestive of a relationship between this hormone and cancer.

In addition to the above-mentioned candidates, genes encoding cytoskeletal proteins were widely represented in prostate and mammary gland studies. This observation can be explained by the nature of the molecules. Indeed, transcription factors and signaling molecules tend to be expressed at low levels, so expression per se or changes in expression may not be detected. On the contrary, cytoskeletal proteins and corresponding mRNAs are much more abundant, simplifying their detection and making changes in their expression levels easy to reveal.

While few genes were common targets of prolactin, a large number of genes were specifically modulated in any given organ, arguing either for a

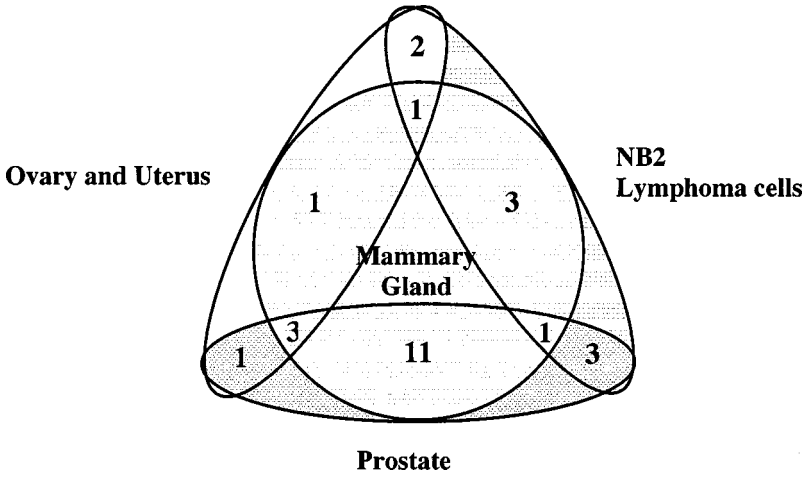

Fig. 1. Venn diagram presenting the total numbers of putative prolactin target genes in different systems. *Note.* Phospolipase genes, and ribosomal proteins, identified as differentially expressed in ovary-uterus, prostate and NB2 lymphoma cells, are not represented here.

tissue-specific expression pattern or for discrepancies between the experimental conditions.

LIMITATIONS AND CAVEATS OF THE GENE EXPRESSION ARRAY APPROACH

The data summarized above have to be considered carefully for various reasons. First of all, different systems were used to undertake the expression profiling. Bole-Feysot *et al.*, for instance, used a lymphoma cell line while the other studies were based on in vivo models. Cell lines may not conserve the expression profiles displayed by their in vivo counterparts. Indeed, recent investigations by Mackay *et al.* (75) on expression profiles in different models where ErbB2 was overexpressed, namely tumor biopsies, BT474 cell lines, and ErbB2-transfected cell lines, showed that there was only one gene, apart from *ErbB2* itself, that was transcribed in an ErbB2-dependent fashion in all of the models. This result clearly argues for substantial variations in transcription profiles between in vivo and in vitro systems.

Moreover, the absence of a gene in a given tissue may simply be explained by the fact that the corresponding probe was not present on the array used for the screen. Indeed, different arrays were used; while Dillner *et al.* selected 3500 clones out of the TIGR rat index, Naylor and colleagues screened 36000 full length mouse genes and ESTs retrieved from the Unigene databank. Obviously, which genes can be identified depends upon the design of the chip or library that was screened, the number of genes arrayed, and the spectrum of functional gene categories. The 91 probes used by Bole-Feysot *et al.* in their differential display experiment were specifically chosen among signaling molecules and transcription factors, with the assumption that expression of these genes was likely to be modulated in response to prolactin action. Hence, the results obtained do not necessarily reflect the overall actual expression profile, but are biased by this arbitrary selection step.

Further discrepancies observed between the genes listed for each experiment may be due to the way the data were analyzed. Arbitrarily, different fold changes were chosen as the thresholds between background signals and signals that were considered relevant for further analysis (1.5-fold for Dillner and colleagues, 10-fold for Brisken *et al.*) (Table IX).

PERSPECTIVES

The present review allowed us to highlight genes modulated in various tissues upon prolactin treatment. Some of these genes may be involved in the potential cancer-promoting role of prolactin. It will be interesting to test whether they can be used as markers in breast, prostate, or ovarian cancers.

Our review also raises the issue of the relevance of microarray data, their validation, and comparison of the results obtained by different groups. Few surveys have been done to assess the accuracy of array data, comparing the results to "laboratory-based

Table IX. Families of Genes Common to the Genomic Screens Outlined in This Paper

Accession #	Title	Annotation	Dillner (prostate)	Robertson (prostate)	Stocco (ovary)	Bole (Nb2)	Baran (uterus)	Naylor (mammary gland)	Brisken (mammary gland)	Ormandy (mammary gland)
AJ131395	Procollagen, type XIV alpha 1	Cell Adhesion								*
M93428	Glycosylation depend. cell adhes.							*		
U03419	Procollagen, type 1, alpha 1			*				*	*	
U32107	Procollagen, type VII alpha 1									*
W75072	Procollagen, type IX, alpha 2								*	
X58251	Procollagen, type 1, alpha 2			*				*	*	
X65582	Procollagen, type VI, alpha 2			*					*	
M81445	Connexin 26	Cell junction						*		*
AF09505	Claudin 3									*
AF087825	Claudin 7									*
U82758	Claudin 5							*		
M20637	Phospholipase C	Signaling			*	*				
J04953	Gelsolin	Cytoskeleton		*					*	
M13805	Keratin complex 1, acidic, gene 17									*
M22832	Keratin complex 1, acidic, gene 18									*
M36120	Keratin complex 1, acidic, gene 19									*
X15662	Keratin complex 2, basic, gene 8		*							*
X04673	Adipsin			*						
X51438	Vimentin		*						*	
	Myosin heavy chain			*		*				
M76131	Elongation factor 2	Elongation factor				*			*	
L29259	Elongation factor SIII				*					
X04480	Insulin-like growth factor I	IGF signalling						*		
AA002605	Insulin-like growth factor II									
L12447	IGF binding protein 5							*		
X81584	IGF binding protein 6							*		
X15358	Insulin-like growth factor binding protein 4								*	
U73744	Heat shock 70	Heat shock							*	
AA163643	Heat shock cognate 71 KD								*	
AA105022	Heat shock protein HSP 90-beta								*	
	Hsp27					*				
	Hsp70					*				
	Hsp86					*				
	HSP60				*					
X60676	HSP47			*						
AK011085	HSPC 307									
	ATP synthase	Metabolism			*	*				
D10215	Casein gamma	Milk protein						*		
L09104	Glucose phosphate isomerase								*	
M10114	Casein kappa							*		*
M36780	Casein alpha							*		*
V00740	Casein delta							*		
X04490	Casein beta							*	*	*
X04673	Adipsin								*	
X93037	WDMN1							*	*	*
X54691	Cytochrome *C* oxidase, subunit IV	Cytochrome *C*			*				*	
J01420	Cytochrome *c* oxidase polypeptide		*							
L09192	Cathepsin D	Cathepsin		*					*	
M36320	Cathepsin H				*					

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Table IX. (*Continued*)

Accession #	Title	Annotation	Dillner (prostate)	Robertson (prostate)	Stocco (ovary)	Bole (Nb2)	Baran (uterus)	Naylor (mammary gland)	Brisken (mammary gland)	Ormandy (mammary gland)
Y00697	Cathepsin L				*					
	b2-microglobulin	Steroidogenesis			*	*				
D10862	Id1	Transcription			*					
AF077861	Helix–Loop–helix inhibitor protein id2	Inhibitor			*			*		
D10864	Id3				*					
V00727	c-Fos	Transcription factor		*		*				
M57683	PDGF receptor alpha	Tyr Kinase		*				*		
X04367	PDGF receptor beta	Receptor						*		
M19681	PDGF-inducible protein (JE) gene							*		

results" using, for instance, Northern Blot or Real-Time PCR approaches. Taniguchi *et al.* (76) showed that microarrays, obtained by designing their own arrays with selected mouse ESTs, and Northern Blot analyses gave globally consistent results, except for 4 out of 46 genes, their fold change being too low to be detected by the array. Similarly, Rajeevan and colleagues (77) demonstrated that genes identified by DNA array with a two- to fourfold difference in expression levels could not be accepted as true or false positive without confirmation by Real-Time PCR approaches. Microarray analysis may therefore overlook genes of significant interest because they display deceivingly small differences in expression levels.

Indeed, as mentioned above, for many signaling molecules such as transcription factors, small changes in expression can have significant biological impact. Furthermore, there is the general problem of microarrays, that all the regulatory changes at protein levels are ignored. It is not clear to date whether protein expression correlates with the transcription level, as measured on arrays. In fact, preliminary surveys by the National Cancer Institute to evaluate expression levels of the corresponding protein products indicate that they correlate in less than 50% of the cases (see http://www.cancer.gov/tarp). This argues that proteomics will be more helpful for understanding of the molecular mechanisms involved in differentiation, carcinogenesis, and other biological processes. Overall these new technologies, such as comparative expression profiling using polysome-bound mRNA, yield a better representation of the proteome than expression profiling employing total mRNA (see issue of *Journal of Mammary Gland Biology and Neoplasia*, Vol. 7, October 2002, for more detailed information).

The development of widely accepted and applicable standards, such as the MIAME (Minimal information on Microarray Experiment), that will enable meaningful comparison of array data between different research groups, the development of uniform validation methods, and a more complete understanding of how to compare and contrast results derived by different techniques, are therefore of great interest (78).

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