

Identification of genes regulated by an interaction between $\alpha_v\beta_3$ integrin and vitronectin in murine decidua

S. S. Mangale^A, D. N. Modi^B and K. V. R. Reddy^{A,C}

^ADepartment of Immunology, National Institute for Research in Reproductive Health (ICMR), J. M. Street, Parel, Mumbai 400012, India.

^BDepartment of Stem Cell Biology, National Institute for Research in Reproductive Health (ICMR), J. M. Street, Parel, Mumbai 400012, India.

^CCorresponding author. Email: shrichi@rediffmail.com

Abstract. The delicate balance between embryo invasion and suppression of maternal immune rejection requires a fully functional decidua in species with haemochorial placenta. Our understanding of the decidual function is very limited due to the molecular and cellular complexity involved in decidualisation. The cell adhesion molecule $\alpha_v\beta_3$ integrin and its ligand vitronectin are upregulated in the mouse decidua during mid-pregnancy. The implications of interactions between $\alpha_v\beta_3$ and vitronectin in regulating decidual function are not known. In the present study, interactions between $\alpha_v\beta_3$ and vitronectin in the decidual cells of the mouse were blocked *in vitro* and effects on cell fate were evaluated by studying the differentially regulated genes by cDNA array and real-time polymerase chain reaction (PCR). The results indicate that expression of various genes involved in apoptotic and cell cycle pathways, as well as cytokine receptors, was deranged. Signalling through $\alpha_v\beta_3$ seems to be important to maintain a balance between cell proliferation and apoptosis, along with the modulation of inflammatory responses of decidual cells.

Additional keywords: cDNA array, mouse, pregnancy.

Introduction

Decidualisation is characterised by proliferation and differentiation of endometrial stromal cells into specialised polyploid cells (decidual cells) and finally the programmed death of decidual cells to accommodate the growing embryo. A fully developed decidua is fundamental to continued survival of the embryo and successful pregnancy in species with haemochorial placentation, because it regulates trophoblast invasion and prevents maternal allograft rejection. Common obstetric diseases, such as pre-eclampsia, intrauterine growth retardation, preterm birth and recurrent pregnancy loss, are known to be associated with abnormal or impaired placental and/or decidual function (Dekker and Sibai 1998; Shih and Kurman 2002). Although there is considerable information regarding decidual morphology, the definitive molecular mechanisms underlying decidual function remain to be resolved. Identification of molecules associated with decidualisation may provide valuable insights into decidual function and the maintenance of pregnancy.

Several studies have suggested a role for integrins and their ligands in decidual function. It has been proposed that altering integrin–matrix interactions may hamper decidualisation (Strakova *et al.* 2003). Endometrial matrix shows enhanced expression of several extracellular matrix (ECM) molecules, such as fibronectin, laminin and collagen IV, during decidualisation, which is also accompanied by switching on of the respective receptor integrins in the transforming decidual cells

(Qin *et al.* 2003). Among these, integrin $\alpha_v\beta_3$ and its ligands vitronectin and osteopontin have generated special interest. This receptor–ligand group is upregulated in the decidualised stroma during pregnancy (Fazleabas *et al.* 1997; Mangale and Reddy 2007). Knocking out the β_3 -subunit in mice results in intrauterine growth retardation, placental defects and reduced embryo survival (Hodivala-Dilke *et al.* 1999), thus highlighting the role of $\alpha_v\beta_3$ in the maintenance of pregnancy.

Our previous studies demonstrated increased expression of $\alpha_v\beta_3$ integrin and its receptor vitronectin in the mouse decidua capsularis at 8.5 days post coitus (d.p.c.), when decidualisation is at its peak (Mangale and Reddy 2007). These *in vivo* observations were indicative of some functional relevance of the $\alpha_v\beta_3$ –vitronectin interaction in decidualisation. On the basis of results from our previous studies and reports demonstrating altered gene transcription and activation of various signalling events in response to binding of $\alpha_v\beta_3$ to its ligands, such as vitronectin and osteopontin, the present study was undertaken to identify the genes that are activated by an interaction between $\alpha_v\beta_3$ and vitronectin in mouse decidual cells. To this end, the interaction between $\alpha_v\beta_3$ and vitronectin was blocked and transcriptional changes were investigated using cDNA expression arrays spotted with 588 genes for various functional pathways, such as apoptosis, cell cycle regulation, intracellular signalling, tumour suppressor pathway, cytokine pathways and transcription factors.

Materials and methods

Animals

The study was approved by the Institutional Ethics Committee for Animal Experimentation. Mature (6–8-week-old) Swiss mice were maintained under a constant photoperiod (14 h light and 10 h dark) and temperature (22–24°C) in the animal house facility of the National Institute for Research in Reproductive Health. Mice were paired for mating overnight. The next morning, mice were checked for vaginal plugs. The day a vaginal plug was found was considered 0.5 d.p.c. of pregnancy. Mice (six in each group) were killed on 6.5, 8.5 and 13.5 d.p.c. to obtain decidua.

Immunohistochemistry

Immunolocalisation of β_3 integrin and vitronectin was performed as described previously (Reddy and Meherji 1999). Sections were deparaffinised in xylene, rehydrated through an alcohol series in distilled water, quenched in 0.3% H_2O_2 in 0.01 M phosphate-buffered saline (PBS), blocked in 1% lamb serum (Bangalore Genei, Bangalore, India) for 1 h and then incubated with rabbit polyclonal antibodies against the β_3 -subunit of integrin (1 : 30) or vitronectin (1 : 30; Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. Negative controls were incubated with 1% lamb serum. Sections were incubated at room temperature for 1.5 h with goat anti-rabbit-horseradish peroxidase (HRP; 1 : 100; Sigma, St Louis, MO, USA). Detection with diaminobenzidine (DAB) substrate was followed by counterstaining with Delafield's haematoxylin. Sections were observed under an Axioskop 2 plus microscope (Carl Zeiss, Oberkochen, Germany) after mounting in di-butyl phthalate in xylene (DPX) and photographed using a digital still camera (DSC-S75; Sony, Tokyo, Japan). Images were digitised using Photoshop 6.0 software (Adobe Systems, San Jose, CA, USA) for documentation purposes. All chemicals were of analytical grade and were purchased from Merck (Mumbai, India) unless stated otherwise.

Preparation of decidual cells

Decidual capsules were dissected out of the uterus and placed in Dulbecco's Modified Eagles Medium (DMEM; GIBCO BRL, Grand Island, NY, USA). Each capsule was checked for the presence of a normally developed embryo and the embryonic tissue was removed as completely as possible under a dissecting microscope. Only decidual tissue from a healthy embryonic bead was used further in the preparation of decidual cells. The tissue was minced with scissors in DMEM containing 0.1% collagenase (Sigma Chemical) and 0.1% RNase-free-DNase I (Sigma Chemical). Pieces of decidua were incubated in collagenase for 30 min at 37°C, then washed twice in medium and incubated in 0.1% trypsin (Sigma Chemical) in DMEM containing 0.1% DNase. Incubation in trypsin was followed by gentle pipetting for 3 min in siliconised Pasteur pipettes. The tissue digests were filtered through a 40- μ m nylon gauze mesh into DMEM. Cells were centrifuged at 300g and resuspended in DMEM with 0.1% DNase. The purity of the cells was checked by staining for vimentin and was found to be approximately 99%, with less than 1% non-decidual component. Cell viability was assessed

by Trypan blue exclusion and was found to be approximately 97% before culture and approximately 90% after culture. Viable cells were used for further experiments.

In vitro blocking of $\alpha_v\beta_3$ integrin

Decidual cells were pre-incubated in DMEM with 10% fetal calf serum (FCS) for 1 h at 37°C and then divided into two groups. Cells in the control group were incubated with DMEM alone, but cells in the treated group were incubated with anti- β_3 -integrin antibody (20 μ g mL⁻¹; subclone 2C9.G2; BD PharMingen, San Diego, CA, USA) at 37°C for 1 h in DMEM. Both groups were seeded on vitronectin (10 μ g mL⁻¹; BD Biosciences, Franklin Lakes, NJ, USA)-coated 24-well culture plates at a concentration of 10⁶ cells per well and cultured for 12 h in DMEM with 10% fetal bovine serum (GIBCO BRL). In the end, the cells were processed for RNA extraction and cDNA array analysis.

Adhesion assays

Adhesion assays were performed to determine whether the β_3 antibody prevents the binding of $\alpha_v\beta_3$ -expressing cells to vitronectin. Decidual cells (4 × 10⁵) pre-incubated with either PBS, anti- β_3 antibody (20 μ g mL⁻¹) or α_5 -integrin antibody (20 μ g mL⁻¹) (BD PharMingen) were added to the vitronectin-coated plates. After 1 h, plates were washed twice with washing buffer. Cells were fixed with 4% paraformaldehyde (Merck, Mumbai, India) and stained with Crystal Violet (Qualigens Fine Chemicals, Mumbai, India) for 10 min. Plates were measured photometrically at 550 nm after dye solubilisation with Triton-X-100. Differences in binding were considered significant when $P \leq 0.05$ (Student's *t*-test).

Complementary DNA array

Total RNA was extracted from decidual cells using Tripure reagent (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's instructions. Total RNA was converted into cDNA using modified oligo (dT) primer and amplified using the BD SMART PCR cDNA Synthesis Kit (BD Biosciences Clontech, Palo Alto, CA, USA). The amplified cDNA was column purified with a NucleoSpin Extraction kit (BD Biosciences Clontech) and labelled using the BD Atlas SMART probe amplification kit (BD Biosciences Clontech) in the presence of 1.85 × 10⁶ Bq [³²P]-dATP (Board of Radiation and Isotope Technology, Government of India, Hyderabad, India), cDNA synthesis primers (complimentary to the array genes; BD Biosciences Clontech) and 2 units of Klenow fragment at 50°C for 30 min. An equal amount of control and experimental labelled probes were used to hybridise to the Atlas mouse cDNA expression array membrane at 68°C (BD Biosciences Clontech). The next day, washings were performed as per the Atlas protocol and membranes were exposed to a phosphor screen (Fuji-film, Tokyo, Japan) for 12 h. Hybridisation signals were quantified using Atlas Image 2.7 software. Hybridisation signal intensities for genes were subtracted from the background and total signal intensities for the arrays were globally normalised. Signal intensities from control and treated groups were compared and converted into ratios by the software. The hybridisations were performed three times in duplicate. Each

Table 1. Primers used for real-time polymerase chain reaction

Gene	Primer sequence	Product size (bp)	Annealing temperature (°C)
Cyclin D ₃	Forward: 5'-TCA CAG CGA CTG AAG TGG AC-3' Reverse: 5'-AGG GCC TGT CTC AAG CTA CA-3'	200	70
Cyclin F	Forward: 5'-CCA GGT GGC CTA ACA CAG TT-3' Reverse: 5'-AGG GCC TCA CAC ACC ATT AG-3'	164	66
Cyclin G	Forward: 5'-GCT GGC GCT ATC TAT CCT TG-3' Reverse: 5'-GGT CAA ATC TCG GCC ACT TA-3'	112	66
E2F	Forward: 5'-ACT CAG GGC CTA TCC ATG TG-3' Reverse: 5'-TGC TGG AAA GTC TGG CTT CT-3'	199	60
p53	Forward: 5'-GCT TCT CCG AAG ACT GGA TG-3' Reverse: 5'-GTC CAT GCA GTG AGG TGA TG-3'	143	64
Rb1	Forward: 5'-CCT TGA ACC TGC TTG TCC TC-3' Reverse: 5'-GGG CAA GGG AGG TAG ATT TC-3'	191	62
GAPDH	Forward: 5'-GAA ACC TGC CAA GTA TGA TGA C-3' Reverse: 5'-ATT GTC ATA CCA GGA AAT GAG C-3'	199	60

time, RNA obtained from a separate blocking experiment was used. Genes showing a greater than twofold change in expression in all three samples were considered as differentially expressed.

Real-time PCR

In order to validate the results of the cDNA array, the expression of six genes (>10%) that showed differential expression by cDNA array was validated using real-time PCR in three separate experiments. The RNA from control and treated cells was extracted using Tripure reagent (Roche Molecular Biochemicals) and converted into cDNA using AMV Reverse Transcriptase (New England BioLabs, Beverly, MA, USA). The cDNA was further subjected to PCR amplification by the iQTMSYBR green supermix and iCycler real-time PCR detection system (BioRad, Hercules, CA, USA). The amplification conditions were as follows: initial denaturation at 95°C for 3 min followed by 40 cycles of denaturation at 95°C for 30 s, annealing at specific temperatures for 30 s (Table 1) and extension at 72°C for 30 s. Final extension was performed at 72°C for 5 min. The fluorescence emitted for each cycle was collected for the entire 30-s period of the extension step in each cycle. The primers used in the PCR (Table 1) were designed using primer3 output online software (<http://frodo.wi.mit.edu/>). For each primer pair, the reaction efficiency was estimated by amplification of a serial dilution of a mouse decidual cDNA pool over a 10-fold range. The relative levels of cyclin D₃, cyclin F, cyclin G, p53, Rb1 and E2F5 were estimated in relation to GAPDH (a housekeeping gene).

The homogeneity of the PCR amplicons was verified by running the products on 2% agarose gels and also by the melting curve method. All PCR amplifications were performed in duplicate and each experiment was repeated three times to test its reproducibility. Mean threshold cycle values generated in each experiment using the iCycler software (BioRad) were computed and normalised to the housekeeping gene (GAPDH) and were used to calculate cDNA concentrations in the samples. Relative expression ratios were calculated manually using the method of

Pfaffl (2001) and considered statistically significant at $P \leq 0.05$ (unpaired *t*-test).

The profiles of differentially expressed genes (*cyclins D₃, F and G, p53, E2F5 and Rb1*) were evaluated in the developing decidua by real-time PCR analysis. Total RNA was extracted from the decidual tissue on different days (6.5, 8.5 and 13.5 d.p.c.) of pregnancy and subjected to real-time PCR as described previously in the Materials and methods section. The fold change difference was considered to be statistically significant when $P \leq 0.05$ (ANOVA).

Functional analysis

In order to assess the functional relevance of differentially expressed genes, downregulated and upregulated groups were subjected to functional analysis using DAVID Functional Annotation Clustering tool (<http://david.abcc.ncifcrf.gov>). The analysis was performed based on the GO_bp database (Gene Ontology for biological process) and KEGG pathways by selecting appropriate parameters in the tool. The annotation tool was used at highest stringency setting to generate clusters of closely related genes. The statistical significance of each cluster was calculated in terms of *P* values using a modified Fisher's exact test. Each cluster was assigned a group Enrichment Score (E-Score), the geometric mean (on a log scale) of the member's *P* values in a corresponding annotation cluster, to rank its biological significance. Thus, the top-ranked annotation cluster had an E-score ($E \geq 1$) that was consistently higher for its annotation members.

Results

Immunolocalisation of $\alpha_v\beta_3$ integrin and vitronectin in mouse decidual cells

$\alpha_v\beta_3$ Integrin and vitronectin were immunolocalised in decidua at 8.5 d.p.c., reconfirming our previous finding (Mangale and Reddy 2007). The decidua revealed characteristic polyploid

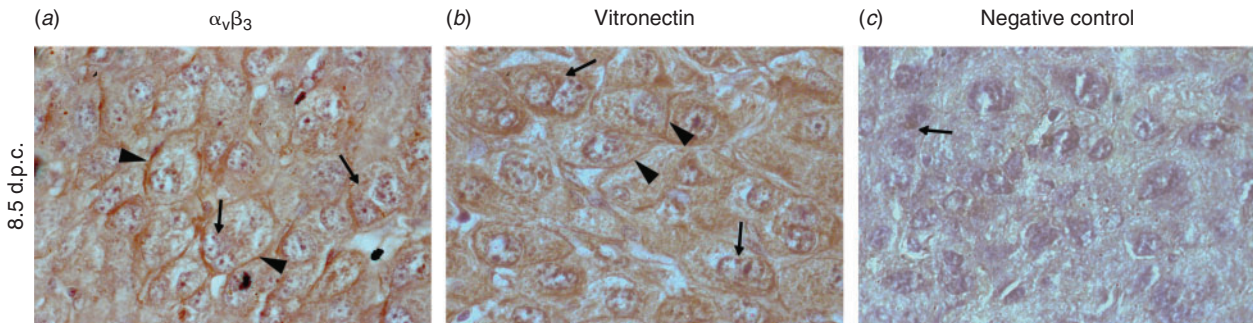


Fig. 1. Expression of (a) β_3 integrin and (b) vitronectin in mouse decidua capsularis on 8.5 days post coitus (d.p.c.). (c) Negative control. Arrows indicate polypliod decidual cells. Arrowheads indicate staining on the cell surface. (Original magnification $\times 100$.)

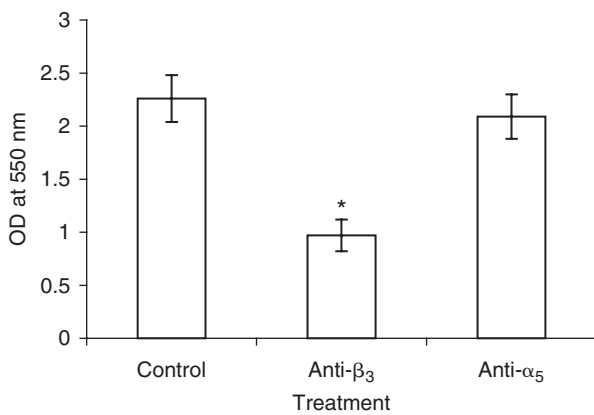


Fig. 2. Effect of the anti- β_3 integrin antibody on binding of decidual cells to vitronectin. Values are the mean \pm s.d. * $P \leq 0.05$ compared with control.

decidual cells with localisation of β_3 -integrin and its ligand vitronectin on the surface and in the cytoplasm of decidual cells (Fig. 1).

Adhesion assay

The adhesion assay revealed that pre-incubation of cells with the anti- β_3 antibody inhibited binding to vitronectin-coated plates. In three independent experiments, the anti- β_3 antibody consistently induced a greater than twofold inhibition compared with control. However, the cells retained their binding capacity when they were incubated with anti- α_5 integrin antibody (Fig. 2).

Complementary DNA array analysis

Expression levels of 588 genes were estimated in decidual cells in which the $\alpha_v\beta_3$ -vitronectin interaction was inhibited. Of the 588 genes spotted on the array, 53 showed differential expression of greater than or equal to twofold between the two groups: 34 genes were upregulated and 19 genes were downregulated in the antibody treated group compared with the control group. The observed differential expression of 53 genes was consistent in three independent experiments.

Upregulated genes

Genes from various families were upregulated when the $\alpha_v\beta_3$ -vitronectin interaction was blocked using a specific antibody (Table 2). The major group comprised the pro-apoptotic genes, TNF- α receptor, APO-1 antigen (FAS) receptor (a member of the tumour necrosis factor (TNF)- α receptor family), interleukin (IL)-converting enzyme (caspase 1), FAS-associated factor 1 (FAF1), UV-excision repair protein (Ras associated with diabetes) (RAD) 21A, RAD 23 and nucleotide diphosphate (NDP) kinase b. Genes like *defender against death 1 (Dad1)*, *Retinoblastoma 1 (Rb1)* and *c-myc* from the oncogene and tumour suppressor family were also upregulated. This was accompanied by upregulation of receptors of the cytokine family, such as granulocyte-macrophage colony stimulating factor receptor (GM-CSFR), transforming growth factor (TGF)- β receptor 1 (TGF β R1), and interferon- γ receptor (IFN- γ R). A large number of transcription factors was upregulated, the most notable being *E2F5*, a transcriptional repressor involved in the cell cycle. Genes from the heat shock protein family and motor proteins were also upregulated. *p27^{kip1}*, an inhibitor of the cell cycle, was also upregulated.

Downregulated genes

The most remarkable downregulation was that of Shc, an adaptor protein that falls under the $\alpha_v\beta_3$ -focal adhesion kinase (FAK)-mediated signalling pathway. Also downregulated was ets-related Proto-oncogene (ELK1) (a member of the erythroblastosis virus E26 oncogene homolog oncogene family), a member of the Shc signalling pathway. Levels of *p53*, a potent regulator of the cell cycle, were also decreased. Interestingly, genes belonging to the cell cycle regulator family *cyclin D3*, *F*, *G1* and *G2* were downregulated, along with *cdc25b* (Table 2).

Validation of array results by real-time PCR

Real-time PCR profiles of the cyclins, *p53*, *Rb1* and *E2F5* in control and antibody treated decidual cells corroborated cDNA array data. Expression levels of cell cycle molecules *cyclin D3*, *F* and *G* and *p53* were less than half in β_3 antibody blocked decidual cells compared with the control group. The expression of *Rb1* and *E2F5* was significantly upregulated in antibody treated decidual cells compared with control (Fig. 3).

Table 2. Differentially regulated genes after inhibition of $\alpha_v\beta_3$ –vitronectin interactions in decidual cells *in vitro*

Rb1, retinoblastoma 1; HSP, heat shock protein; FAF, Fas-associated factor; NDP, nucleotide diphosphate; ICE, interleukin-converting enzyme; RAD, UV-excision repair protein (Ras associated with diabetes); NURR1, transcriptionally inducible nuclear receptor related 1; GM-CSF-R, granulocyte–macrophage colony stimulating factor receptor; TGF- β , transforming growth factor β ; IFN- γ , interferon- γ ; BRCA 1, breast cancer 1

Gene family	Accession no.	Gene name	Fold difference
Upregulated genes			
Oncogenes and tumour suppressors	M26391	Rb1	4.3
	X01023	c-myc proto-oncogene	18
Intracellular kinase network members	M64429	β -raf protooncogene	2.18
Chaperones, heat shock proteins and stress response proteins	M36829	HSP 84	6.4
	M36830	HSP 86	3.8
	U40930	Oxidative stress-induced protein	21
Apoptosis associated proteins	U83628	Defender against cell death (Dad1)	30
	U39643	FAF1	3.3
	M83649	FAS1 receptor, Fas1 antigen	2.8
	X68193	NDP kinase b	4.75
	L28095	ICE caspase-1	4.8
	D49429	RAD 21A (UV excision repair protein)	31
	X92411	RAD 23	10.5
Transcription activators and repressors	M94087	Activating transcription factor 4 (MAT F4)	3.3
	L12721	Delta-like protein precursor	2.46
	S53744	Brain-specific transcription factor, NURR1	6
	S68377	Brn 3.2 POU transcription factor	12
	U36340	Kruppel like factor-3	5.3
	M37163	Caudal type homeobox 1 cdx1	18
	S74520	Caudal type homeobox 2 cdx2	31
	X72310	E2F dimerisation partner 1	29
	X86925	E2F transcription factor 5	26
	M20157	Early growth response	7.25
	L39770	Gbx 2 (globin x-transcription factor)	8.33
	X53476	Non-histone chromosomal protein HMG 14	25
	U25096	Lung Kruppel-like factor	61
Growth factors and chemokine receptors	M31042	T-Lymphocyte-activated protein	12.6
	M85078	GM-CSF-R	12.5
	D25540	TGF- β receptor 1	2.2
	M28233	IFN- γ receptor	2.4
Motor proteins	D26077	Kinesin-like protein KIF	2.5
	U04443	Non-muscle myosin light chain 3	40
Lipid metabolism	D78647	Phospholipase A ₂	2.1
Cdk inhibitors	U10440	p27 kip	2.3
Downregulated genes			
Oncogenes and tumor suppressors	M26391	BRCA 1	5.5
	K01700	Cellular tumour antigen p53	2.8
	U12570	Von Hippel-Lindau	13.14
	X87257	Elk-1, ets-related Proto-oncogene	4.4
	S65038	Gli oncogene, zinc finger transcription factor	14
	M13945	pim-1 proto-oncogene	6
	Z22649	c-mpl	36
	X67735	Mas proto-oncogene	3.75
Adaptors and receptor-associated proteins	U15784	Shc transforming protein	24
Cell cycle proteins	U43844	G ₁ /S-specific cyclin D ₃	3.7
	Z47766	S/G ₂ /M-specific cyclin F	15.5
	Z37110	G ₂ /M-specific cyclin G	9.3
	U95826	G ₂ /M-specific cyclin G ₂	6.25
	S93521	cdc 25b	2.6
Miscellaneous	D17584	β protachykinin a	49
	U03560	HSP 27	3.4
	X81464	Translin	2.28
	X96859	Ubiquitin-conjugating enzyme E2	2.58
	Z46845	Preproglucagon	36

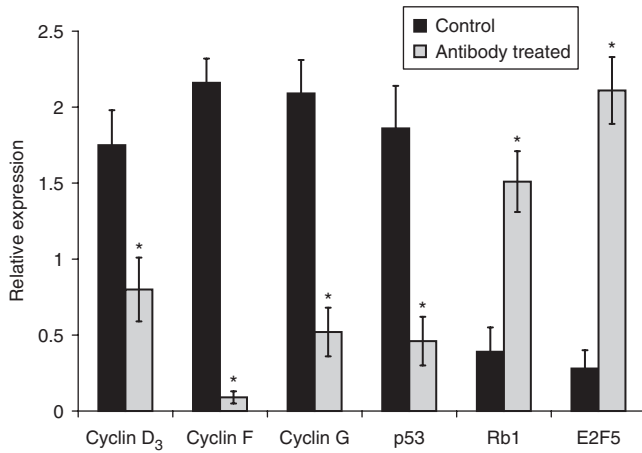


Fig. 3. Validation of array results by real-time polymerase chain reaction *in vitro* on 8.5 days post coitus (d.p.c.). Values show the relative level of expression of the different genes normalised against GAPDH in antibody treated *v.* control. Data are the mean \pm s.d. * $P \leq 0.05$ compared with control.

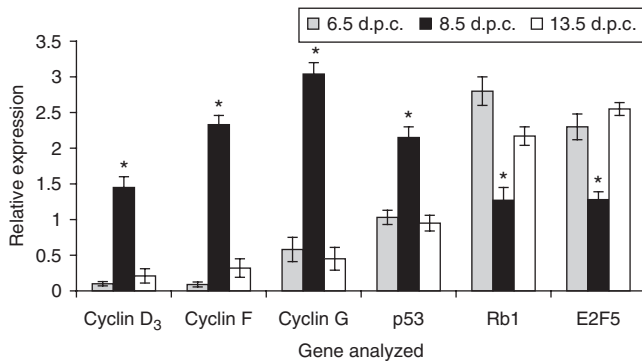


Fig. 4. Analysis of differentially expressed genes in mouse decidua during pregnancy. Values show the relative level of expression of the different genes normalised against GAPDH at 6.5, 8.5 and 13.5 days post coitus (d.p.c.). Data are the mean \pm s.d. * $P \leq 0.05$ compared with 6.5 d.p.c.

Real-time PCR analysis of cyclins, p53, Rb1 and E2F5 in decidua at various embryonic ages

There were dynamic changes in the expression of genes for the cyclins, p53, Rb1 and E2F5 in mouse decidua at different ages. Expression of cyclin D₃, F and G and p53 was maximal and significantly higher at 8.5 d.p.c. compared with that on 6.5 and 13.5 d.p.c. Conversely, expression of Rb1 and E2F5 was significantly lower at 8.5 d.p.c. compared with that on 6.5 and 13.5 d.p.c. (Fig. 4).

Functional annotation

It was evident from functional annotation clustering that most of the upregulated genes clustered into 12 functional groups, which had a significant number of genes ($E \geq 1$). These genes are known to play a role in cellular metabolism, transcriptional regulation or apoptosis (Table 3). Conversely, among the downregulated genes, most significant clusters comprised genes involved in cell cycle regulation and cellular physiological processes.

Discussion

The $\alpha_v\beta_3$ integrin has well-documented roles in various cellular processes, including proliferation, differentiation and apoptosis (Stupack *et al.* 2001; Faccio *et al.* 2003; Sahni and Francis 2004). Because $\alpha_v\beta_3$ and its ligand vitronectin are coexpressed in mouse decidua on the cell surface as well as in the cytoplasm (Fig. 1), it is conceivable that this receptor–ligand interaction may be playing a similar role in decidua function. To further our understanding of this receptor–ligand pair in decidua cell functioning, changes in gene expression profiles in response to $\alpha_v\beta_3$ –vitronectin interactions in mid-gestation murine decidua were investigated. To this end, the $\alpha_v\beta_3$ –vitronectin interaction in decidua cells was blocked by treating cells with an $\alpha_v\beta_3$ integrin-blocking antibody *in vitro* and the expression of 588 genes was assessed by cDNA expression array. Adhesion assays confirmed that $\alpha_v\beta_3$ integrin was blocked and our experimental set up was valid (Fig. 2). Complementary DNA array results revealed that disruption of the vitronectin– $\alpha_v\beta_3$ interaction in mid-trimester decidua cells *in vitro* results in marked changes in expression profiles of genes belonging to multiple pathways, mainly cellular proliferation and apoptosis.

Using the above assay system, we identified several key molecules whose expression was affected by the disruption of the $\alpha_v\beta_3$ –vitronectin interaction. Fifty-three genes (approximately 9%) showed differential expression when the interaction between $\alpha_v\beta_3$ and vitronectin was inhibited (Table 2). Among these, the expression of 34 genes was consistently upregulated and that of 19 genes was consistently downregulated in three independent experiments. The results of cDNA arrays were also validated by real-time PCR, in which the expression of cyclins D₃, F and G and p53 was found to be downregulated, whereas that of E2F5 and Rb1 was found to be upregulated after $\alpha_v\beta_3$ blockade. Although in quantitative terms the fold change observed was not in complete agreement, the pattern of differential expression was found to be similar between the cDNA array and real-time PCR. Similar quantitative discrepancies between the data derived by real-time PCR and cDNA arrays have been reported previously (Rajeevan *et al.* 2001; Andersen *et al.* 2004). These discrepancies have been explained by differences in the sensitivity of the two methods, the dynamic range of detectors and the mode of assessment (hybridisation *v.* amplification).

The biological significance of the differentially expressed genes was assessed using DAVID functional annotation software. It was evident that most of the upregulated genes belonged to clusters involved in cellular metabolism, transcriptional regulation and apoptosis, whereas the downregulated genes fell in two significant clusters that included genes involved in cell cycle regulation and cellular physiological processes. Thus, it appears that $\alpha_v\beta_3$ –vitronectin signalling directly or indirectly regulates the expression of some molecules involved in these pathways to modulate decidua functions. Indeed, among the downregulated genes, *Shc1* and *Elk-1* are known to be involved in $\alpha_v\beta_3$ -mediated signalling pathway for the regulation of cell proliferation (Wary *et al.* 1996; Aplin *et al.* 2001). These genes, along with those for cyclins D₃, F, G₁ and G₂, were clustered under the group of cell cycle regulators. Cyclin D₃, cyclin G₁ and G₂ are known to be present in high levels in proliferating decidua cells during

Table 3. Functional annotation clustering of differentially expressed genes

Cluster	Biological process	No. genes involved	%	E-score
Upregulated genes				
1	Primary metabolism	27	77.14	4.93
2	Regulation of transcription	15	42.86	4.62
3	Apoptosis	8	22.86	4.13
4	Positive regulation of transcription	5	14.29	2.91
5	Cellular physiological process	31	88.57	2.63
6	Positive regulation of transcription from RNA polymerase II promoter	4	11.43	1.99
7	Localisation of cell	4	11.43	1.75
8	Response to protein stimulus	3	8.57	1.55
9	Neuron differentiation	4	11.43	1.44
10	Lymphocyte differentiation	3	8.57	1.44
11	Negative regulation of cellular physiological process	5	14.29	1.38
12	DNA repair	3	8.57	1.23
13	Cellular protein metabolism	10	28.57	0.94
14	Intracellular transport	4	11.43	0.61
15	Immune response	4	11.43	0.45
Downregulated genes				
1	Regulation of cell cycle	10	58.82	6.59
2	Mitosis	5	29.41	4.84
3	Regulation of cellular physiology	3	17.65	0.96
4	Metabolism	5	29.41	0.55
5	Regulation of transcription	3	17.65	0.19

mid-pregnancy (Das *et al.* 1999; Yue *et al.* 2005), when decidual proliferation is at its peak (Abrahamsohn and Zorn 1993; Correia-da-Silva *et al.* 2004), whereas cyclin F-null mice fail to survive owing to defects in placentation (Tetzlaff *et al.* 2004). Taken together, these data suggest that, during mid-pregnancy, proliferation of decidual cells requires the coordinated activity of cyclins, which are possibly regulated by the $\alpha_v\beta_3$ –vitronectin interaction in decidual tissue.

Cyclins are proximal members of the cell cycle regulation assembly, which, in turn, activates or suppresses downstream pathways that comprise molecules like Rb1, E2F5 and p53 (Sears and Nevins 2002). Functional annotation clustering of the upregulated genes identified a cluster that was comprised of the genes *Rb1* and *E2F5*, which are involved in the negative regulation of the cell cycle (Table 3). Rb1 is a well-studied anti-proliferative molecule involved in cell cycle regulation (Kato 1999), whereas E2F transcription factors are placed furthest downstream in the cell cycle regulatory mechanism. Of the E2F family, E2F5 is known for transcriptional repression (Sears and Nevins 2002). Upregulation of Rb1 and E2F5, along with cell cycle inhibitor p27^{Kip1}, as indicated by cDNA array analysis further strengthens our hypothesis that blocking of $\alpha_v\beta_3$ leads to inhibition of cell cycle events in the decidua. Complementary DNA array and real-time PCR analysis also indicated downregulation of p53. Increased levels of p53 have been reported in *in vitro* decidualised human endometrial stromal cells and are speculated to be of relevance in the decidua (Pohnke *et al.* 2004). However, it remains to be investigated whether this change in p53 expression

is a result of direct transcriptional regulation by $\alpha_v\beta_3$ or through cyclins.

Because inhibition of the cell cycle was the most striking effect observed after interactions between $\alpha_v\beta_3$ and vitronectin had been blocked and the genes for cyclins D₃, F and G₁, p53, Rb1 and E2F5 occupy different niches in the cell cycle regulatory machinery, the expression profiles of these genes were validated by real-time PCR. The results of the PCR were in agreement with those from cDNA array (Fig. 3). To gain further insight into the regulation of these molecules, we investigated the expression profile of these genes (cyclin D₃, cyclin F, cyclin G₁, p53, Rb1 and E2F5) during the proliferative (6.5 and 8.5 d.p.c.) and regressive (13.5 d.p.c.) phases in the decidua (Fig. 4). The results show that, at 8.5 d.p.c., when decidual proliferation has reached its peak, the expression of cyclins D₃, F and G₁ and p53 was also highest, whereas the expression of the cell cycle inhibitory molecules Rb1 and E2F5 was lowest. The expression levels coincided with those of $\alpha_v\beta_3$ and vitronectin. Conversely, in the regressive phase, at 13.5 d.p.c., when the levels of $\alpha_v\beta_3$ and its ligands have diminished, the expression of the cyclins and p53 dropped, with a concomitant increase in the mRNA levels for Rb1 and E2F5. These observations collectively support our hypothesis that cell proliferation in the decidua may be regulated, in part, by interactions between $\alpha_v\beta_3$ and vitronectin.

Functional annotation clustering placed the pro-apoptotic genes *FAS*, *caspase-1*, *c-myc*, *RAD 21*, *RAD 23* and *NDP kinase b* in a single group with high statistical significance (Table 3), indicating a shift towards apoptosis. This was further supported by

the upregulation of genes representing receptors for the inflammatory cytokines *TGF- β 1* and *IFN- γ* , which promote apoptosis in endometrial stromal cells *in vitro* (Moulton 1994; Christian *et al.* 2001; Chatzaki *et al.* 2003). Together, these data leads us to postulate that the $\alpha_v\beta_3$ -vitronectin interaction may be crucial for maintaining the balance between cell proliferation and apoptosis in decidua and, when this interaction is perturbed, pro-apoptotic pathways predominate in decidual cells.

The decidua is known to undergo sequential proliferation, differentiation and death from implantation to the end of pregnancy. Initial attachment of the embryo to the uterus triggers proliferation of endometrial stromal cells, followed by their transformation into decidual cells. During the course of embryonic invasion, the matrix metalloproteinases secreted by the trophoblast cells degrade the ECM, thereby perturbing integrin-ECM interactions. On the basis of the present findings, it may be envisaged that the complex interplay between $\alpha_v\beta_3$ integrin and vitronectin, or a perturbation in their interaction caused by matrix degrading enzymes produced by the embryo, is crucial for the dynamic balance between proliferation and death in the decidua. This may be crucial for controlling embryonic invasion, a disturbance that may lead to overinvasion or termination of the pregnancy. To the best of our knowledge, this is the first study that identifies the putative pathways driven by ECM-integrin interactions in murine decidual cells. Establishing the role of the downstream targets of the $\alpha_v\beta_3$ -vitronectin interaction under *in vivo* conditions may further unravel the intricacies of decidual function.

Acknowledgements

The present study was funded by the Indian Council of Medical Research. The authors are grateful to Dr Chander P. Puri (Director, NIRRH) for support and encouragement. SSM was supported by the Council for Scientific and Industrial Research (India) with a Senior Research Fellowship.

References

- Abrahamsohn, P. A., and Zorn, T. M. T. (1993). Implantation and decidualization in rodents. *J. Exp. Zool.* **266**, 603–628. doi:10.1002/JEZ.1402660610
- Andersen, C. L., Jensen, J. L., and Ørntoft, T. F. (2004). Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Res.* **64**, 5245–5250. doi:10.1158/0008-5472.CAN-04-0496
- Aplin, A. E., Stewart, S. A., Assoian, R. K., and Juliano, R. L. (2001). Integrin-mediated adhesion regulates ERK nuclear translocation and phosphorylation of Elk-1. *J. Cell Biol.* **153**, 273–281. doi:10.1083/JCB.153.2.273
- Chatzaki, E., Kouimtoglou, E., Margioris, A. N., and Gravanis, A. (2003). Transforming growth factor β 1 exerts an autocrine regulatory effect on human endometrial stromal cell apoptosis, involving the FasL and Bcl-2 apoptotic pathways. *Mol. Hum. Reprod.* **9**, 91–95. doi:10.1093/MOLEHR/GAG011
- Christian, M., Marangos, P., Mak, I., Mcvey, J., Barker, F., White, J., and Brosens, I. J. (2001). Interferon- γ modulates prolactin and tissue factor expression in differentiating human endometrial stromal cells. *Endocrinology* **142**, 3142–3151. doi:10.1210/EN.142.7.3142
- Correia-da-Silva, G., Bell, S. C., Pringle, J. H., and Teixeira, N. A. (2004). Patterns of uterine cellular proliferation and apoptosis in the implantation site of the rat during pregnancy. *Placenta* **25**, 538–547. doi:10.1016/J.PLACENTA.2003.11.007
- Das, S. K., Lim, H., Paria, B. C., and Dey, S. K. (1999). Cyclin D₃ in the mouse uterus is associated with the decidualization process during early pregnancy. *J. Mol. Endocrinol.* **22**, 91–101. doi:10.1677/JME.0.0220091
- Dekker, G. A., and Sibai, B. M. (1998). Etiology and pathogenesis of preeclampsia: current concepts. *Am. J. Obstet. Gynecol.* **179**, 1359–1375. doi:10.1016/S0002-9378(98)70160-7
- Faccio, R., Takeshita, S., Zallone, A., Patrick, R. F., and Teitelbaum, S. L. (2003). c-Fms and the $\alpha_v\beta_3$ integrin collaborate during osteoclast differentiation. *J. Clin. Invest.* **111**, 749–758. doi:10.1172/JCI200316924
- Fazleabas, A. T., Bell, S. C., Fleming, S., Sun, J., and Lessey, B. A. (1997). Distribution of integrins and the extracellular matrix proteins in the baboon endometrium during the menstrual cycle and early pregnancy. *Biol. Reprod.* **56**, 348–356. doi:10.1095/BIOLREPROD56.2.348
- Hodivala-Dilke, K. M., McHugh, X., Tsakiria, D. A., Rayburn, H., Crowley, M., Ullman-Cullre, E. P., Ross, B. S., Collier, B. S., Teitelbaum, T., and Hynes, R. O. (1999). β_3 integrin deficient mice are a model for Glanzman's thrombasthenia showing placental defects and reduced survival. *J. Clin. Invest.* **103**, 229–238.
- Kato, J. Y. (1999). Induction of S phase by G1 regulatory factors. *Front. Biosci.* **4**, d787–792. doi:10.2741/KATO
- Mangale, S. S., and Reddy, K. V. R. (2007). Expression pattern of integrins and their ligands in mouse feto-maternal tissues during pregnancy. *Reprod. Fertil. Dev.* **19**, 452–460. doi:10.1071/RD06143
- Moulton, B. C. (1994). Transforming growth factor- β stimulates endometrial stromal apoptosis *in vitro*. *Endocrinology* **134**, 1055–1060. doi:10.1210/EN.134.3.1055
- Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **29**, e45. doi:10.1093/NAR/29.9.E45
- Pohnke, Y., Schneider-Merck, T., Fahnenstich, J., Kempf, R., Christian, M., Milde-Langosch, K., Brosens, J. J., and Gellersen, B. (2004). Wild-type p53 protein is up-regulated upon cyclic adenosine monophosphate-induced differentiation of human endometrial stromal cells. *J. Clin. Endocrinol. Metab.* **89**, 5233–5244. doi:10.1210/JC.2004-0012
- Qin, L., Wang, Y. L., Bai, S. X., Ji, S. H., Qiu, W., Tang, S., and Piao, Y. S. (2003). Temporal and spatial expression of integrins and their extracellular matrix ligands at the maternal-fetal interface in the rhesus monkey during pregnancy. *Biol. Reprod.* **69**, 563–571. doi:10.1095/BIOLREPROD.103.015198
- Rajeevan, M. S., Vernon, S. D., Taysavang, N., and Unger, E. R. (2001). Validation of array-based gene expression profiles by real-time (kinetic) RT-PCR. *J. Mol. Diagn.* **3**, 26–31.
- Reddy, K. V. R., and Meherji, P. K. (1999). Integrin cell-adhesion molecules in endometrium of fertile and infertile women throughout the menstrual cycle. *Indian J. Exp. Biol.* **37**, 323–331.
- Sahni, A., and Francis, C. W. (2004). Stimulation of endothelial cell proliferation by FGF-2 in the presence of fibrinogen requires $\alpha_v\beta_3$. *Blood* **104**, 3635–3641. doi:10.1182/BLOOD-2004-04-1358
- Sears, R. C., and Nevins, J. R. (2002). Signaling networks that link cell proliferation and cell fate. *J. Biol. Chem.* **277**, 11 617–11 620. doi:10.1074/JBC.R100063200
- Shih, I. E. M., and Kurman, R. J. (2002). Molecular basis of gestational trophoblastic diseases. *Curr. Mol. Med.* **2**, 1–12. doi:10.2174/1566524023362960
- Strakova, Z., Szmidt, M., Srisuparp, S., and Fazleabas, A. T. (2003). Inhibition of matrix metalloproteinases prevents the synthesis of insulin-like growth factor binding protein-1 during decidualization in the baboon. *Endocrinology* **144**(12), 5339–5346. doi:10.1210/EN.2003-0471
- Stupack, D. G., Puente, X. S., Boutsaboualoy, S., Storgard, C. M., and Cheresch, D. A. (2001). Apoptosis of adherent cells by recruitment of caspase-8 to unligated integrins. *J. Cell Biol.* **155**, 459–470. doi:10.1083/JCB.200106070

- Tetzlaff, M. T., Bai, C., Finegold, M., Wilson, J., Harper, J. W., Mahon, K. A., and Elledge, S. J. (2004). Cyclin F disruption compromises placental development and affects normal cell cycle execution. *Mol. Cell. Biol.* **24**, 2487–2498. doi:10.1128/MCB.24.6.2487-2498.2004
- Wary, K. K., Mainiero, F., Isakoff, S. J., Marcantonio, E. E., and Giancotti, F. G. (1996). The adaptor protein Shc couples a class of integrins to the control of cell cycle progression. *Cell* **87**, 733–743. doi:10.1016/S0092-8674(00)81392-6
- Yue, L., Daikoku, T., Hou, X., Li, M., Wang, H., Nojima, H., Dey, S. K., and Das, S. K. (2005). Cyclin G1 and cyclin G2 are expressed in the periimplantation mouse uterus in a cell-specific and progesterone-dependent manner: evidence for aberrant regulation with Hoxa-10 deficiency. *Endocrinology* **146**, 2424–2433. doi:10.1210/EN.2004-1605

Manuscript received 10 September 2007, accepted 15 November 2007