

# The POU homeodomain protein OCT3 as a potential transcriptional activator for fibroblast growth factor-4 (FGF-4) in human breast cancer cells

Peixiang WANG\*, Donald R. BRANCH\*†‡§, Meenakshi BALI\*†‡§, Gilbert A. SCHULTZ||, Paul E. GOSS\*† and Tianru JIN\*†‡<sup>1</sup>

\*Department of Medicine, University of Toronto, 67 College Street, Toronto, ON, Canada M5G 2M1, †Department of Laboratory Medicine and Pathobiology, University of Toronto, 67 College Street, Toronto, ON, Canada M5G 2M1, ‡Division of Cell and Molecular Biology, Toronto General Research Institute, University Health Network, 67 College Street, Toronto, ON, Canada M5G 2M1, §Canadian Blood Services, Toronto Center, Toronto, ON, Canada M5G 2M1, and ||Department of Biochemistry and Molecular Biology, University of Calgary, Health Sciences Centre, 3330 Hospital Drive N.W., Calgary, AB, Canada T2N 4N1

The POU (representing a homeodomain protein family of which the founder members are Pit-1, Oct-1/2 and Unc-86) homeodomain protein OCT3/Oct-3 (where OCT stands for octamer-binding protein) is an embryonic transcription factor expressed in oocytes, embryonic stem and embryonic carcinoma cells. We have demonstrated previously that human breast cancer cells regain the ability to express OCT3 mRNA [Jin, Branch, Zhang, Qi, Youngson and Goss (1999) *Int. J. Cancer* **81**, 104–112]. Antibodies against human OCT3 were not available when this study was conducted. By using a human OCT3–glutathione S-transferase fusion protein to affinity purify a polyclonal antibody against the mouse Oct-3, we obtained an antibody that enabled us to detect OCT3 in human breast cancer cells by Western-blot analysis. Thus we have now confirmed that OCT3 is expressed in human breast cancer cells but not in normal human breasts and in three other organs. When breast cancer cell lines were treated with all-*trans*-retinoic acid, OCT3 expression was repressed, associated with decreased cell proliferation. Although

another POU protein Brn-3 has been shown to be a repressor for BRCA1 (breast-cancer susceptibility gene 1), OCT3 does not repress human or mouse BRCA1/Brca-1 promoters. However, OCT3 is capable of activating a fusion promoter containing the fibroblast growth factor-4 (FGF-4) enhancer element. In addition, we documented for the first time that human breast cancer cells express FGF-4 protein, and its expression could be inhibited by all-*trans*-retinoic acid. Furthermore, overexpressing OCT3 stimulated endogenous FGF-4 expression in MCF7 breast cancer cell line. These observations indicate that OCT3 protein is selectively expressed in human breast cancer cells, and its expression may be implicated in mammary gland tumorigenesis via up-regulating FGF-4 expression.

**Key words:** all-*trans*-retinoic acid (ATRA), breast cancer, fibroblast growth factor-4 (FGF-4), octamer-binding protein (OCT3)/Oct-3, Pit-1, Oct-1/2, and Unc-86 (POU).

## INTRODUCTION

De-regulated expression of certain homeodomain (HD) proteins, encoded by homeobox genes, may lead to the development of leukaemias, lymphomas and other solid tumours [1,2]. Using several approaches, we have examined previously expressions of members of the POU (representing a homeodomain protein family of which the founder members are Pit-1, Oct-1/2 and Unc-86) HD protein [also known as the octamer- or octamer-binding protein (OCT)-binding transcription factor] family in human mammary gland epithelial cells. We found that human breast cancer cells express at least four POU genes: the ubiquitous transcription factor OCT1; the B lymphocyte-specific transcription factor OCT2; a POU gene isolated from rat skin and mouse thymus OCT11 (Skn-1a/i); and an embryonic transcription factor OCT3 [3]. Among these four POU genes, OCT3 and OCT11 expressions were only detected in the malignant cells, but not in normal mammary gland epithelia cultures. Previously, the POU gene Brn-3b has also been found to be overexpressed in breast cancer cells, and was found to be capable of repressing the expression of the mouse breast-cancer susceptibility gene 1 (Brca-1) promoter [4].

OCT3/Oct-3 is an embryonic transcription factor [5–7]. Oct-3 expression is essential for the identity of the pluripotential founder cell population in the mammalian embryo. In rodents, Oct-3 was found to be expressed only in oocytes, embryonic stem (ES) and embryonic carcinoma (EC) cells, but not in ES or EC cells treated with retinoic acid [8,9] or in any adult organs. Our observations, therefore, suggest that breast cancer cells regain the ability to express this embryonic transcription factor.

In the present study, we generated an affinity-purified anti-OCT3 antibody for examining OCT3 expression in human breast cancer cells by Western-blot analysis and by electrophoretic mobility-shift assay (EMSA). Using this antibody, we demonstrate in the present study that OCT3 is, indeed, selectively expressed in human breast cancer cells and OCT3 expression can be down-regulated by treating the cells with all-*trans*-retinoic acid (ATRA), a differentiation promoting anti-tumour agent. In addition, we show in the present study, for the first time, that human breast cancer cells express the fibroblast growth factor-4 (FGF-4) protein. OCT3 is not capable of repressing the expression of either human or mouse BRCA1/Brca-1 gene promoters. However, OCT3 co-transfection activates a reporter promoter fused

Abbreviations used: ATRA, all-*trans*-retinoic acid; BE, breast epithelia; BRCA1, breast-cancer susceptibility gene 1; EC, embryonic carcinoma; EMSA, electrophoretic mobility-shift assay; ES, embryonic stem; FGF-4, fibroblast growth factor-4; FE, FGF-4 enhancer; GST, glutathione S-transferase; HD, homeodomain; LUC, luciferase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; OCT, octamer-binding protein; POU, Pit-1, Oct-1/2 and Unc-86; TK, thymidine kinase.

<sup>1</sup> To whom correspondence should be addressed, at the Division of Cell and Molecular Biology, Toronto General Research Institute (e-mail tianru.jin@utoronto.ca).

with the FGF-4 enhancer (FE) element, and overexpressing OCT3 induces up-regulation of endogenous FGF-4 expression.

## EXPERIMENTAL

### Cell lines and cell culture

All the cell lines were obtained from the A.T.C.C. (Manassas, VA, U.S.A.). The non-malignant MCF10A cell line was maintained according to the manufacturer's instructions. Two normal human breast epithelial cell cultures, namely NB1 and NB2, were purchased from Clonetics (San Diego, CA, U.S.A.).

### Antibody purification and Western-blot analysis

A polyclonal antibody against the mouse Oct-3 (=Oct-4) was generated previously by immunizing New Zealand White rabbits using a 21-amino-acid peptide identical with a C-terminal portion of mouse Oct-3 [9]. Human OCT3A cDNA was kindly provided by Dr Graeme Bell [10]. It was then subcloned into a cytomegalovirus-promoter-driven expression vector pBAT7 [11]. OCT1 cDNA was a gift from Dr Wiship Herr [12]. Plasmid constructs for glutathione S-transferases (GSTs) fusion proteins, containing the full-length human OCT3 and a C-terminal portion of OCT3, were generated by inserting the corresponding OCT3 cDNA into a pGEX-4T-2 vector. The procedure for purifying the anti-mouse Oct-3 antibody with the GST-OCT3-fusion protein was that of Bar-Peled and Raikhel [13]. The human BRCA1 and the mouse Brca-1 promoter luciferase (LUC) reporter gene constructs were provided by Dr David Johnson [14] and by Dr Bernard Futscher [15] respectively.

Western-blot analysis was performed as described previously [16]. Protein concentrations of cell lysates and nuclear extracts were quantified by means of the bicinchoninic acid assay (Pierce, Rockford, IL, U.S.A.). To ensure equal loading, membranes were treated with Ponceau S stain and the protein bands were visualized before Western immunoblotting. Anti FGF-4 antibody (C-18) and the FGF-4 blocking peptide (sc-1361 P) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.).

### EMSA and super-shifting

The DNA sequence for the wild-type octamer-binding probe is 5'-GATCCTGATTGATTWGCATGTCCA-3'. The core motif in this probe is ATTWGCAT (W = A or T). The DNA sequence for the mutant octamer-binding probe is 5'-GATCCTGATTG-GTTSGCGGTGTCCA-3' (S = G or C). Methods for nuclear extraction, EMSA and super-shifting assay were described previously [3,11].

### Proliferation assay

Proliferation assay was conducted by an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] method described previously using the 96-well plates [17]. Briefly, 6000 cells from an indicated cell line were distributed into each well with a vehicle (95 % ethanol) or with an indicated amount of ATRA. At different time intervals, cell numbers (viable) in each well were determined by adding the dye MTT and a colorimetric measurement was made.

### Cell transfection, LUC reporter gene analysis and inducible OCT3 expression

NIH-3T3 cells were transfected by a calcium precipitation method, whereas MCF7 cells were transfected by either an elec-

troporation method [11] or by LIPOFECTAMINE™ (Invitrogen Life Technology, Carlsbad, CA, U.S.A.). Unless specified, LUC reporter gene analyses were conducted 16 h after transfection [11]. OCT-TK-LUC (where TK stands for thymidine kinase) is an LUC reporter gene plasmid in which one copy of the octamer-binding motif [3] was inserted at the 5'-end of the TK promoter [11]. FE-TK-LUC was constructed by inserting one copy of the human FE element in front of the TK promoter in the TK-LUC fusion gene construct [11]. DNA sequence for the FE element is 5'-GATCTGAAAACCTCTTTGTTTGGATGCTAATGGGATAC-TTAAAG-3'. The method of ecdysone-inducible mammalian expression system (Invitrogen Life Technology) was utilized for overexpressing OCT3 in the MCF7 cell line. Briefly, human OCT3A cDNA was inserted into the Pind-inducible expression vector, downstream of a minimal heat-shock promoter and the modified ecdysone response element. The expression of OCT3 is then controlled by a heterodimeric nuclear receptor, encoded by a plasmid namely pVgRXR, in the presence of ponasterone A as the inducer. Pind-OCT3 and pVgRXR were co-transfected into the MCF7 cell line by LIPOFECTAMINE™ (Invitrogen Life Technology) in the absence of serum for 12 h. Cells were then grown in serum-containing medium. Ponasterone A was added either immediately or 12 h later, and then the cells were harvested 24 h after growing in serum-containing medium for Western-blot analysis.

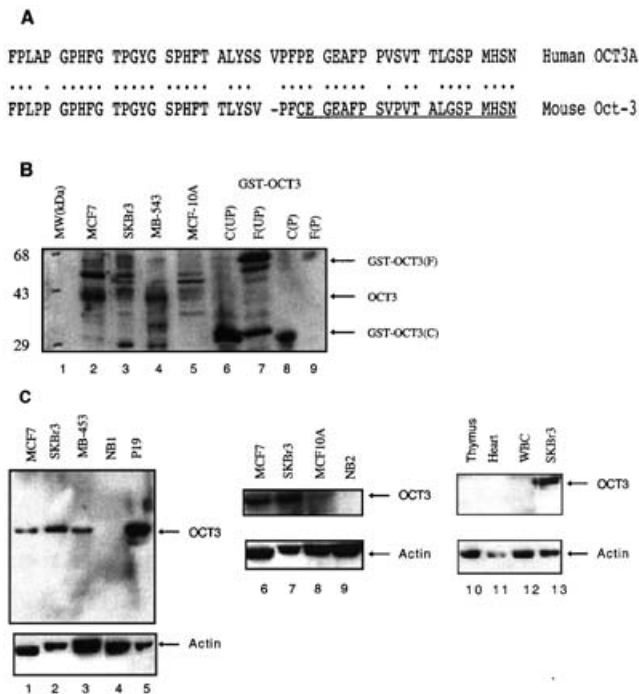
## RESULTS

### Detection of OCT3 expression by Western-blot analysis

The polyclonal antibody against mouse Oct-3 has been used to detect this transcription factor in rodent species [9]. This antibody was raised against a C-terminal portion of the mouse Oct-3 [9] (Figure 1A). We found that this antibody could also detect a GST-fusion protein containing either the full-length or a C-terminal portion of human OCT3 (Figure 1B, lanes 6–9). This antibody recognized a number of proteins from a battery of human breast cancer cell lines, including one that was approx. 40 kDa in mass (Figure 1B, lanes 2–5). To improve the specificity of the antibody, we made a GST-fusion protein containing a C-terminal portion of human OCT3 (Figure 1A). This C-terminal region shares 91 % amino acid sequence identity with the corresponding motif in the mouse Oct-3 [18]. This GST-fusion protein was then used to purify the polyclonal anti-mouse Oct-3 antibody [13]. The purified antibody only recognizes a 40 kDa protein from three human breast cancer cell lines examined (Figure 1C, lanes 1–3) and it co-migrates with a protein that is abundantly expressed in the P19 EC cell line (cf. lane 5). We therefore assumed that this protein represents human OCT3. OCT3 expression was not detected in two normal human breast epithelial cell cultures (NB1 and NB2, Figure 1, lanes 4 and 9) and not in three normal human tissues examined (heart, thymus and peripheral white blood cells, lanes 10–12). Furthermore, OCT3 expression is detectable, although much less abundant, in the non-malignant MCF10A cell line (lane 8). This is consistent with our previous observation that MCF10A cells express OCT3 mRNA at a low level, detectable by reverse transcriptase-PCR [3].

### OCT3 expression is down-regulated by ATRA

OCT3/Oct-3 expression in the mouse EC and ES cells and in the human Tera2 EC cell line could be down-regulated by ATRA [6,9]. When the MCF7 and SKBr3 cell lines were treated with ATRA, OCT3 expression was substantially inhibited at 24 h, and was barely detectable after 48 h (Figure 2A). This inhibition was found



**Figure 1** Examination of OCT3 expression in human breast cancer cells by Western-blot analysis

(A) Comparison of a 49-amino-acid C-terminal portion of human OCT3 [10] with that in mouse Oct-3 [9]. Amino acid residues for the peptide utilized in generating the polyclonal antibody are underlined. cDNA encoding of the 49-amino-acid peptide motif from human OCT3 was utilized for making a GST-fusion protein, GST-OCT3 (C). (B) Western-blot analysis using the unpurified anti-OCT3 antibody against whole cell lysates of four human BE cell lines (lanes 2–5), unpurified and glutathione–Sephadex purified GST-OCT3 proteins (lanes 6–9). C(UP) and F(UP), unpurified GST-OCT3 proteins; C(P) and F(P), purified GST-OCT3 proteins. C and F represent the C-terminal portion and full-length respectively. (C) Western-blot analysis using the affinity-purified anti-OCT3 antibody. NB1 and NB2, two normal human breast epithelial cultures purchased from Clonetics. P19, a mouse EC cell line. Nuclear proteins (25  $\mu$ g) from each cell line were loaded. For three normal human tissues, 50  $\mu$ g of total proteins were utilized. Protein integrity was assayed by a re-probing procedure with an antibody against actin. WBC, white blood cells.

to be associated with the decreased cell proliferation, examined by MTT analysis (Figure 2B).

We have isolated a variant ATRA-resistant cell line of SKBr3 by continuing to grow the parental cell line in an ATRA-containing medium. Cell proliferation of this variant, namely SKBr3 (V), cannot be effectively inhibited by ATRA (Figure 2B, right panel). OCT3 expression in this variant also cannot be substantially inhibited by ATRA at 24 h (Figure 2A). At 48 h, OCT3 expression is still detectable by Western-blot analysis (but not by EMSA, see below). Interestingly, at 72 h, OCT3 expression was found to be restored to its initial level (Figure 2A).

We have established previously the methodology to examine the expression of POU or OCT proteins in human breast cancer cells by EMSA, using a DNA probe containing an octamer-binding site [3]. The DNA–protein complexes detected in human breast epithelia (BE) were designated as OCT-BEs. Among the complexes detected, OCT-BE1 was found to contain the ubiquitous transcription factor OCT1 [3]. With the affinity purified anti-OCT3 antibody, we conducted the super-shifting analysis to investigate which complex contains OCT3. We found that the complex OCT-BE5 could be super-shifted by the OCT3 antibody (Figure 3, cf. lanes 2 and 6). The super-shifted complex was observed for the SKBr3 (v) cells not treated with ATRA (lane 6), or the cells treated with ATRA for 24 h (lane 7). After a 48 h

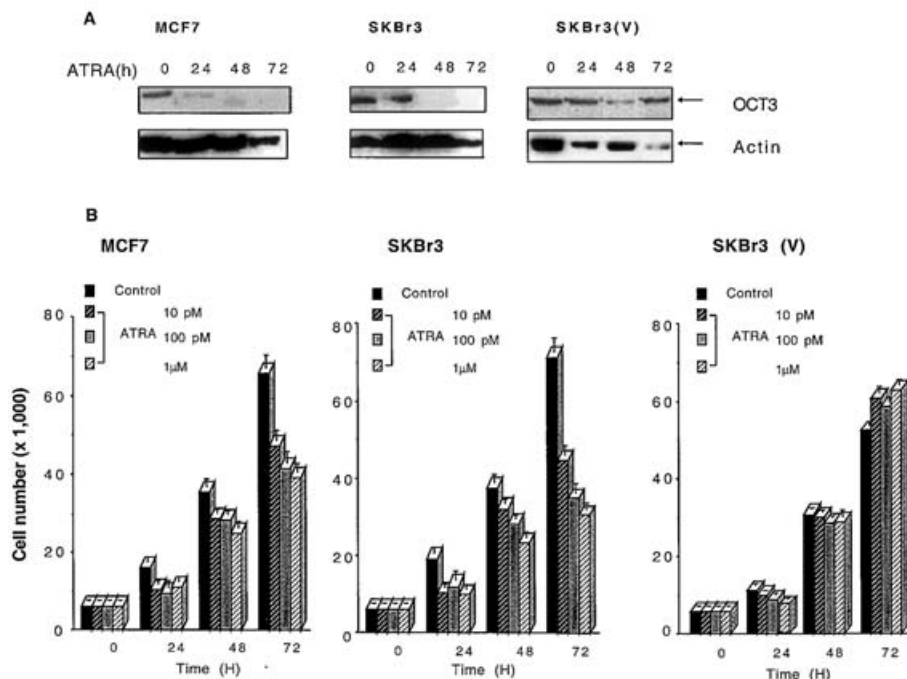
ATRA treatment, the super-shifted complex was undetectable (lane 8), and it re-appeared after a 72 h treatment (lane 9). Such a result is consistent with the Western-blot result against this variant cell line (Figure 2A, right panel). Interestingly, the formation of OCT-BE3 was substantially reduced 24 h after ATRA treatment (lane 3). The identity of this complex is yet to be determined.

### OCT3 activates FE element–TK fusion promoter

Budhram-Mahadeo et al. [4] found that the expression of another POU protein Brn-3b is also increased in human breast cancer cell lines, and it is able to repress substantially the expression of the BRCA1 promoter. We examined the effect of OCT3 cDNA transfection on a LUC reporter gene driven by four human BRCA1 promoter constructs [15] of different sizes and the mouse Brca-1 promoter [14]. As shown in Figure 4(A), OCT3 transfection generated no substantial repression or activation on either the human or the mouse BRCA1/Brca-1 promoters. To verify that the OCT3 cDNA expression construct generated in the present study could act as a transcription factor, we made an OCT–TK–LUC fusion gene construct in which one copy of the octamer-binding site was inserted at the 5'-end of the TK promoter. The activity of this OCT–TK fusion promoter is comparable with that of the parental TK promoter when transfected into the NIH-3T3 and MCF7 cell lines (Figure 4B). However, when OCT3 cDNA was co-transfected with the OCT–TK fusion promoter, more than a 3-fold activation was observed (Figure 4B), indicating that OCT3 cDNA utilized in the present study is able to act as a transcriptional activator. Therefore BRCA1/Brca-1 may not be a direct target of OCT3.

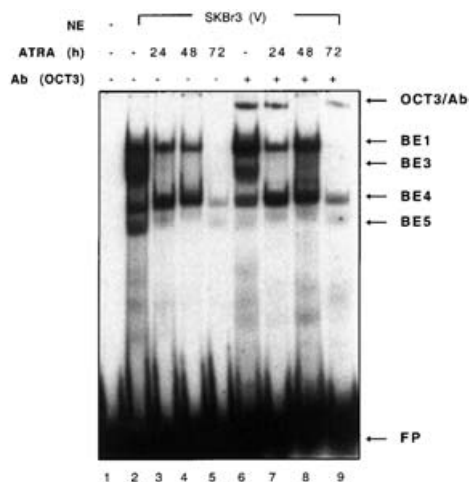
In the mouse EC cell line P19, OCT-3 has been shown to bind and activate the FGF-4 promoter through an enhancer element [20]. Transfection of the wild-type FGF-4 into the malignant cell line MCF7 generates novel MCF7 cell lines which show rapid growth and spontaneous metastasis in ovariectomized and tamoxifen-treated nude mice [21], whereas transfection of FGF-4 into the non-malignant cell line HBL100 led to tumorigenic conversion of this cell line [22]. However, so far the expression of endogenous FGF-4 in human breast cancer cells has not been detected [23]. To investigate whether human breast cancer cell lines express FGF-4, we conducted an examination of FGF-4 expression in human mammary gland epithelial cell lines by Western-blot analysis. As shown in Figure 5, FGF-4 could be detected in four breast cancer cell lines examined (lanes 1–4). In the non-malignant MCF10A cell line (lane 5) and two normal human breast epithelial cell cultures (lanes 7 and 8), FGF-4 expression was not detectable. To confirm further that the 16 kDa protein detected does represent FGF-4, we preabsorbed the antibody with the FGF-4 blocking peptide. The preabsorbed antibody is no longer able to detect this 16 kDa protein (results not shown).

To initiate an examination whether OCT3 is capable of activating FGF-4 in human breast cancer cells, we made an FE and TK fusion promoter, in which one copy of the FGF-4 FE [20] was inserted 5'-end of the TK promoter. This fusion promoter was approx. seven times more active compared with the parental TK promoter itself when it was transfected into the MCF7 cell line [6]. Co-transfection of OCT3 was found to activate further the fusion promoter by approx. 3-fold (Figure 6). This activation is dependent on the FE element, because it was not observed for the parental TK promoter. In addition, this activation is OCT3-specific because it was not observed when OCT3 was replaced by OCT1 (Figure 6). Although OCT1 does not activate FE–TK–LUC, the same OCT1 expression vector has been found to activate the promoter for the caudal homeobox gene Cdx-2, reported by our group previously [24].



**Figure 2** Effect of ATRA on OCT3 expression and cell proliferation

(A) Breast cancer cell lines MCF7, SKBr3 and an ATRA-resistant variant of SKBr3 [SKBr3 (V)] were treated with ATRA (1  $\mu$ M final concentration). At indicated time intervals, cells were harvested and nuclear extracts were prepared. Nuclear proteins (25  $\mu$ g) were loaded for Western-blot analysis. The same membrane was stripped and re-probed with an anti-actin antibody. (B) Six thousand cells from an indicated cell line were incubated with a vehicle (95% ethanol) or an indicated amount of ATRA (10 pM to 1  $\mu$ M). At different time intervals, numbers for the viable cells were determined by the MTT analysis. Data are expressed as means ( $n=8$ )  $\pm$  S.E.M.



**Figure 3** Examination of OCT3 expression in SKBr3 (V) by EMSA and super-shifting

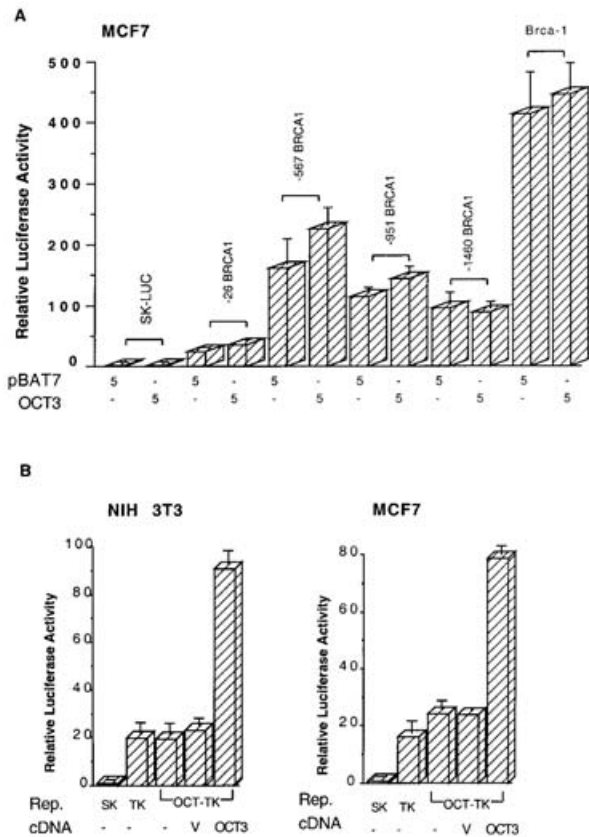
Nuclear proteins (5  $\mu$ g) from ATRA-treated or ATRA-untreated SKBr3 variant cells [SKBr3 (V)] were utilized. Purified anti-Oct3 antibody (2  $\mu$ g) was applied for the super-shifting analysis. OCT3/Ab, super-shifted complex; FP, free probe. Designations of these DNA-protein complexes were reported previously [3].

We then examined the relationship between OCT3 expression and the FE-TK-LUC activity by conducting a transfection assay with an ATRA-treated MCF7 cell line. Expressions of both OCT-TK and FE-TK-LUC were substantially inhibited by ATRA, especially after 72 h, whereas the expression of the parental TK-

LUC was only slightly inhibited (Figure 7A). In addition, ATRA repressed the expressions of both endogenous OCT-3 and FGF-4 in the MCF7 cell line (Figure 7B). Finally, we examined the effect of overexpressing OCT3 on the expression of endogenous FGF-4 in human breast cancer cells. Co-transfecting the MCF7 cells with the empty Pind expression vector and pVgRXR generated no substantial effect on the expression of either OCT3 or FGF-4, in the presence or absence of ponasterone A (Figure 7C, lanes 1 and 2). Co-transfecting the MCF7 cells with Pind-OCT3 and pVgRXR in the absence of ponasterone A also generated no effect on the expression of OCT3 and FGF-4 (Figure 7C, lane 3). In the presence of ponasterone A for 12 h, OCT3 expression, but not FGF-4 expression was found to be enhanced (Figure 7C, lane 4). Treating the cells with ponasterone A for 24 h stimulated the expressions of both OCT3 and FGF4 (Figure 7C, lane 5).

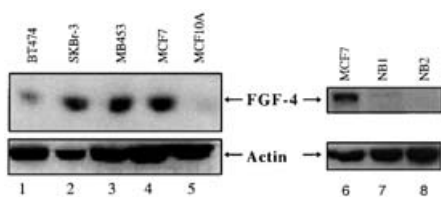
## DISCUSSION

Abnormal expressions of members of different families of HD proteins are attributed to the development of various tumours [1,2]. A given HD protein may up-regulate the expression of a growth factor or repress the expression of a tumour suppressor [25,26]. The expression of several families of HD proteins in mammary glands has been previously examined by several laboratories [27-34]. We demonstrated that four POU genes are expressed in human breast epithelial cells. Among them, OCT3 and OCT11 can only be detected in the malignant mammary gland epithelial cells [3]. The expression of another POU protein, Brn-3b, is also de-regulated in human breast cancer cells. Brn-3b may repress human BRCA1 promoter, detected by a LUC reporter gene analysis [4].



**Figure 4** OCT3 transfection does not repress human BRCA1 or mouse Brca-1 promoter

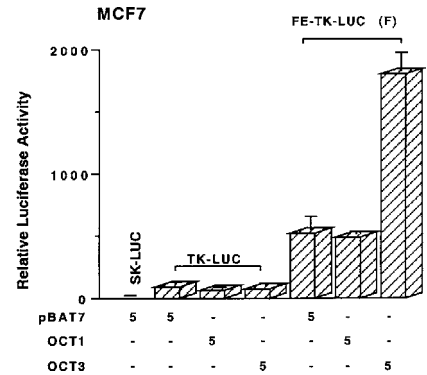
(A) SK-LUC, a promoterless LUC construct. Four different-sized human BRCA1 promoter constructs [15] and the mouse Brca-1 promoter [14] were examined. The indicated reporter plasmid (5  $\mu$ g) was co-transfected with 5  $\mu$ g of cDNA. Data are expressed as mean relative LUC activity ( $n=3$ )  $\pm$  S.E.M. normalized to (1) the activity obtained after transfection of the SK-LUC in the same experiment and (2) total protein utilized in each assay. (B) OCT3 transfection activates a TK promoter with a 5'-end located wild-type octamer-binding site in the NIH-3T3 and MCF7 cell lines. Indicated LUC reporter plasmid (5  $\mu$ g) was transfected into the indicated cell line with 5  $\mu$ g of OCT3A cDNA driven by a cytomegalovirus promoter (OCT3) or a control vector (pBAT7, V). SK, a promoterless control; TK, the LUC reporter gene driven by a TK promoter; OCT-TK, a wild-type octamer-binding site motif was inserted 5'-end of the TK promoter. Data are expressed as mean relative LUC activity ( $n=3$ )  $\pm$  S.E.M. as mentioned in (A).



**Figure 5** Examination of FGF-4 expression in human breast cancer cell lines by Western-blot analysis

Total proteins (25  $\mu$ g) were loaded for Western-blot analysis. The same membrane was stripped and re-probed with anti-actin antibody.

OCT3/Oct-3 is an embryonic transcription factor identified in mammals [6,18,35,36]. The terms Oct-3, Oct-4 and Oct-3/Oct-4 have been utilized to describe the same gene in rodents in literature. In the present study, we use the terms OCT3 and Oct-3 for this gene in humans and rodents respectively. This is based on



**Figure 6** OCT3 activates an FE element-TK promoter

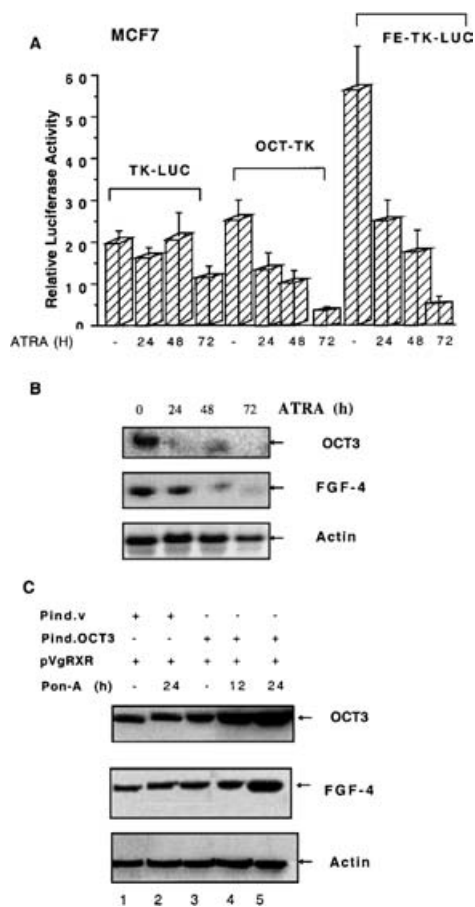
SK-LUC, a promoterless LUC reporter gene construct; TK, a reporter gene construct in which the expression of the LUC reporter is driven by a TK promoter; FE-TK-LUC (F), one copy FE element was inserted 5'-end of the TK promoter in the TK-LUC fusion gene construct. The indicated reporter gene (5  $\mu$ g) was co-transfected with 5  $\mu$ g of indicated cDNA. Data are expressed as mean relative LUC activity ( $n=3$ )  $\pm$  S.E.M. as mentioned in caption to Figure 4(A).

the nomenclature regulations for *HOX/Hox* genes [37]. In rodent species, Oct-3 is only expressed in oocytes, ES and EC cells. Inducing differentiation of the ES or EC cells by retinoic acid results in down-regulated transcription of Oct-3 [19,38].

Our previous observation suggests that human breast cancer cells regain the ability to express OCT3 [3]. To extend further these observations to the protein level, we purified the anti-mouse Oct-3 antibody using a GST-fusion protein, containing a C-terminal portion of the human OCT3 protein. By using this antibody, we demonstrated clearly that OCT3 is expressed in all breast cancer cell lines examined, but not in two normal mammary gland epithelial cell cultures and three normal human tissues. We do find that this antibody can detect a trace amount of OCT3 in the non-malignant MCF10A cell line. This is inconsistent with our previous observation that MCF10A cell line expresses a very small amount of OCT3 mRNA [3].

An important observation made in the present study is that OCT3 expression in breast cancer cells could be down-regulated by ATRA. Down-regulated OCT3 expression by ATRA in MCF7 and SKBr3 cell lines is associated with decreased cell proliferation. Furthermore, we found that in an ATRA-resistant SKBr3 variant, OCT3 expression cannot be consistently and effectively inhibited by ATRA. Since ATRA has been found to down-regulate Oct-3 transcription in rodents [8,9], we suggest that in human breast cancer cells, OCT3 may also serve as one of the mediators for ATRA in up-regulating genes implicated in cell proliferation and/or down-regulating genes implicated in differentiation or apoptosis. However, we cannot eliminate the possibility that down-regulated OCT3 expression by ATRA could be attributed to an indirect mechanism. To examine the detailed mechanisms for down-regulation of human OCT3 expression by ATRA and other retinoids, it is necessary to isolate and characterize the human OCT3 gene promoter.

Although another POU protein Brn-3b was shown to repress BRCA1 promoter, based on our results, we were able to conclude that OCT3 does not possess this repressive function. To date three downstream target genes for OCT3/Oct-3 have been identified. In the EC cells, Oct-3 is capable of up-regulating FGF-4 [39,40] and the promoter for platelet-derived growth factor  $\alpha$ -receptor gene [19]. In the JA<sub>r</sub> choriocarcinoma cells, Oct-3 is capable of down-regulating the expression of the  $\beta$  subunit of human chorionic gonadotropin [41]. Transfection of FGF-4 into the breast cancer cell line MCF7 was found to generate novel cell



**Figure 7** Examination of the effect of OCT3 on FGF-4 expression

(A) ATRA represses FE-TK-LUC expression. MCF7 cells were transfected with 5  $\mu$ g of indicated LUC fusion gene plasmid. ATRA (1  $\mu$ M final concentration) was added 12, 36 and 60 h after the transfection, and cells were harvested for LUC assay 84 h after the transfection. In this way, times for which cells were treated with ATRA were 24, 48 and 72 h respectively. Data are expressed as mean relative LUC activity ( $n=3$ )  $\pm$  S.E.M. as mentioned in the legend to Figure 4(A). (B) ATRA represses the expression of both OCT3 and FGF-4. MCF7 cells were treated with 1  $\mu$ M ATRA for varied times. At indicated time intervals, cells were harvested and total protein was extracted. Total proteins (25  $\mu$ g) were loaded for Western-blot analysis against OCT3 and FGF-4. The same membrane was stripped and re-probed with an anti-actin antibody. (C) Over-expressing OCT3 activates endogenous FGF-4 expression. MCF7 cells were transfected by the method of LIPOFECTAMINE™ with either empty Pind vector (Pind.v) and pVgRXR, or Pind.OCT3 and pVgRXR in serum-free medium for 12 h. Cells were then grown in serum-containing medium for 24 h before harvesting for Western-blot analysis. Total proteins (25  $\mu$ g) were loaded for Western-blot analysis. The same membrane was stripped and re-probed with an anti-actin antibody.

lines that are more tumorigenic and metastatic in ovariectomized and tamoxifen-treated athymic mice [21]. In addition, transfection of FGF-4 into the non-malignant HBL100 cell line was found to produce novel cell lines that are tumorigenic [22]. However, the biological significance of FGF-4 expression in mammary gland tumorigenesis has not been adequately evaluated. This is because the expression of endogenous FGF-4 in breast cancer cell lines has not been detected by reverse transcriptase-PCR [23]. This failure to detect FGF-4 mRNA might be due to its relatively low expression level, primer design and/or the PCR conditions utilized. In the present study, we show for the first time that FGF-4 protein is expressed in four breast cancer cell lines examined, but is not expressed to an appreciable level in the non-malignant MCF10A cell line. It has been demonstrated previously that Oct-3 and Sox2 bind to adjacent sites on the FGF-4-enhancer

element and synergistically activate FGF-4 promoter [20,42]. We found that this 42 bp native enhancer element activates the TK promoter more than 7-fold in the MCF7 breast cancer cell line. In contrast, a 24 bp synthetic OCT-binding element cannot activate the same TK promoter, although both fusion promoters could be activated by OCT3 transfection. This result would suggest that the native FE element is active in human breast cancer cells. In addition, overexpressing OCT3 resulted in enhanced expression of endogenous FGF-4 in the MCF7 cell line. Given that increased levels of FGF-4 have been previously associated with breast epithelial carcinogenesis [21,22], it is possible that OCT3 up-regulated FGF-4 expression has potential clinical relevance. Thus whether OCT3 is involved in the initiation and progression of mammary gland carcinogenesis via activating the expression of FGF-4 requires further examinations.

In summary, we demonstrated for the first time the detection of OCT3 expression at the protein level, and that FGF-4 is expressed in human breast cancer cells. In addition, we find that OCT3 expression in human breast cancer cells, similar to its rodent homologue in EC cells, could be down-regulated by ATRA and that down-regulated OCT3 expression is associated with inhibited cell growth and down-regulation of FGF-4 expression. Finally, our results suggest that de-regulated expression of POU genes in human breast cancer cell lines may not only repress the expression of a tumour suppressor [4], but also activate the expression of an oncogenic growth factor.

We thank Dr Bernard W. Futscher (Department of Pharmacology and Toxicology, University of Arizona, Tucson, AZ, U.S.A.) and Dr David G. Johnson (Department of Carcinogenesis, University of Texas, M. D. Anderson Cancer Center, Smithville, TX, U.S.A.) for providing BRCA1/Brcal LUC fusion gene constructs; Dr Winship Herr (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, U.S.A.) and Dr Graeme I. Bell (Department of Medicine, University of Chicago, Chicago, IL, U.S.A.) for providing OCT1 and OCT3 cDNAs. The present study is partially supported by the University of Toronto Dean's Fund, the Grant Miller Cancer Research grant and a feasibility grant from Canadian Breast Cancer Research Initiative to T.J.

## REFERENCES

- Boncinelli, E. (1997) Homeobox genes and disease. *Curr. Opin. Genet. Dev.* **7**, 331–337
- Shimamoto, T., Ohyashiki, K., Toyama, K. and Takeshita, K. (1998) Homeobox genes in hematopoiesis and leukemogenesis. *Int. J. Hematol.* **67**, 339–350
- Jin, T., Branch, D. R., Zhang, X., Qi, S., Youngson, B. and Goss, P. E. (1999) Examination of POU homeobox gene expression in human breast cancer cells. *Int. J. Cancer* **81**, 104–112
- Budhram-Mahadeo, V., Ndisang, D., Ward, T., Weber, B. I. and Latchman, D. S. (1999) The Brn-3b POU family transcription factor represses expression of the BRCA-1 anti-oncogene in breast cancer cells. *Oncogene* **18**, 6684–6691
- Scholer, H. R., Balling, R., Hatzopoulos, A. K., Suzuki, N. and Gruss, P. (1989) Octamer binding proteins confer transcriptional activity in early mouse embryogenesis. *EMBO J.* **8**, 2551–2557
- Okamoto, K., Okazawa, H., Okuda, A., Sakai, M., Muramatsu, M. and Hamada, H. (1990) A novel octamer binding transcription factor is differentially expressed in mouse embryonic cells. *Cell (Cambridge, Mass.)* **60**, 461–472
- Ma, Y. G., Rosfjord, E., Huebert, C., Wilder, P., Tiesman, J., Kelly, D. and Rizzino, A. (1992) Transcriptional regulation of the murine k-FGF gene in embryonic cell lines. *Dev. Biol.* **154**, 45–54
- Pikarsky, E., Sharif, H., Ben-Shushan, E. and Bergman, Y. (1994) Retinoic acid represses Oct-3/4 gene expression through several retinoic acid-responsive elements located in the promoter-enhancer region. *Mol. Cell. Biol.* **14**, 1026–1038
- Wang, L. and Schultz, G. A. (1996) Expression of Oct-4 during differentiation of murine F9 cells. *Biochem. Cell Biol.* **74**, 579–584
- Takeda, J., Seino, S. and Bell, G. I. (1992) Human Oct3 gene family: cDNA sequences, alternative splicing, gene organization, chromosomal location, and expression at low levels in adult tissues. *Nucleic Acids Res.* **20**, 4613–4620
- Jin, T. and Drucker, D. J. (1996) Activation of proglucagon gene transcription through a novel G1 promoter element by the caudal-related homeodomain protein cdx-2/3. *Mol. Cell. Biol.* **16**, 19–28

- 12 Cleary, M. A. and Herr, W. (1995) Mechanisms for flexibility in DNA sequence recognition and VP16-induced complex formation by the Oct-1 POU domain. *Mol. Cell. Biol.* **15**, 2090–2100
- 13 Bar-Peled, M. and Raikhel, N. V. (1996) A method for isolation and purification of specific antibodies to a protein fused to the GST. *Anal. Biochem.* **241**, 140–142
- 14 Wang, A., Schneider-Broussard, R., Kumar, A. P., Macleod, M. C. and Johnson, D. G. (2000) Regulation of BRCA1 expression by the Rb-E2F pathway. *J. Biol. Chem.* **275**, 4532–4536
- 15 Rice, J. C., Massey-Brown, K. S. and Futscher, B. W. (1998) Aberrant methylation of the BRCA1 CpG island promoter is associated with decreased BRCA1 mRNA in sporadic breast cancer cells. *Oncogene* **17**, 1807–1812
- 16 Branch, D. R. and Mills, G. B. (1995) pp60<sup>c-src</sup> expression is induced by activation of normal human T lymphocytes. *J. Immunol.* **154**, 3678–3685
- 17 Branch, D. R. and Guilbert, L. J. (1986) Differential expression of tumor necrosis factor- $\alpha$  isoforms from lipopolysaccharide- and cytokine-stimulated mouse macrophages. *Int. J. Biochem. Cell Biol.* **28**, 949–955
- 18 Rosner, M. H., Vignano, M. A., Ozato, K., Timmons, P. M., Poirier, F., Rigby, P. W. J. and Staudt, L. M. (1990) A POU-domain transcription factor in early stem cells and germ cells of the mammalian embryo. *Nature (London)* **345**, 686–692
- 19 Kraft, H. J., Mosselman, S., Smits, H. A., Hohenstein, P., Piek, E., Chen, Q., Artzt, K. and Van Zoelen, E. J. (1996) Oct-4 regulates alternative platelet-derived growth factor  $\alpha$  receptor gene promoter in human embryonal carcinoma cells. *J. Biol. Chem.* **271**, 12873–12878
- 20 Ambrosetti, D. C., Scholer, H. R., Dailey, L. and Basilico, C. (2000) Modulation of the activity of multiple transcriptional activation domains by the DNA binding domains mediates the synergistic action of Sox2 and Oct-3 on the fibroblast growth factor-4 enhancer. *J. Biol. Chem.* **275**, 23387–23397
- 21 Mcleskey, S. W., Kurebayashi, J., Honig, S. F., Zwiebel, J., Lippman, M. E., Dichson, R. B. and Kern, F. G. (1993) Fibroblast growth factor 4 transfection of MCF7 cells produces cell lines that are tumorigenic and metastatic in ovariectomized or tamoxifen-treated athymic nude mice. *Cancer Res.* **53**, 168–177
- 22 Soultou, B., Gamby, C., Crepin, M. and Hamelin, R. (1996) Tumoral progression of human breast epithelial cells secreting FGF2 and FGF4. *Int. J. Cancer* **68**, 675–681
- 23 Penault-Llorca, F., Bertucci, F., Adelaide, J., Parc, P., Coulier, F., Jacquemier, J., Birnbaum, D. and Delapeyriere, O. (1995) Expression of FGF and FGF receptor genes in human breast cancer. *Int. J. Cancer* **61**, 170–176
- 24 Jin, T. and Li, H. (2001) Pou homeodomain protein OCT1 is implicated in the expression of the caudal-related homeobox gene Cdx-2. *J. Biol. Chem.* **276**, 14752–14758
- 25 Care, A., Silvani, A., Meccia, E., Mattia, G., Stoppacciaro, A., Parmiani, G., Peschle, C. and Colombo, M. P. (1996) HOXB7 constitutively activates basic fibroblast growth factor in melanomas. *Mol. Cell. Biol.* **16**, 4842–4851
- 26 Stuart, E. T., Haffner, R., Oren, M. and Gruss, P. (1995) Loss of p53 function through PAX-mediated transcriptional repression. *EMBO J.* **14**, 5638–5645
- 27 Chariot, A. and Castronovo, V. (1996) Detection of HoxA1 expression in human breast cancer. *Biochem. Res. Commun.* **222**, 292–297
- 28 Chariot, A., Moreau, L., Senterre, G., Sobel, M. E. and Castronovo, V. (1995) Retinoic acid induces three newly cloned HoxA1 transcripts in MCF7 breast cancer cells. *Biochem. Biophys. Res. Commun.* **215**, 713–720
- 29 Chen, F. and Capecchi, M. R. (1999) Paralogous mouse Hox genes, Hoxa9, Hoxb9, and Hoxd9, function together to control development of the mammary gland in response to pregnancy. *Proc. Natl. Acad. Sci. U.S.A.* **96**, 541–546
- 30 Friedmann, Y., Daniel, C. A., Strickland, P. and Daniel, C. W. (1994) Hox genes in normal and neoplastic mouse mammary gland. *Cancer Res.* **54**, 5981–5985
- 31 Friedmann, Y. and Daniel, C. W. (1996) Regulated expression of homeobox genes Msx-1 and Msx-2 in mouse mammary gland development suggests a role in hormone action and epithelial–stromal interactions. *Dev. Biol.* **177**, 347–355
- 32 Andres, A. C. and Strange, R. (1999) Apoptosis in the estrous and menstrual cycles. *J. Mammary Gland Biol. Neoplasia* **4**, 221–228
- 33 Phippard, D. J., Weber-Hall, S. J., Sharpe, P. T., Naylor, M. S., Jayatalake, H., Maas, R., Woo, I., Roberts-Clark, D., Francis-West, P. H., Liu, Y. H. et al. (1996) Regulation of Msx-1, Msx-2, Bmp-2 and Bmp-4 during fetal and postnatal mammary gland development. *Development* **122**, 2729–2737
- 34 Srebrow, A., Friedmann, Y., Ravanpay, A., Daniel, C. W. and Bissell, M. J. (1998) Expression of Hoxa-1 and Hoxb-7 is regulated by extracellular matrix-dependent signals in mammary epithelial cells. *J. Cell. Biochem.* **69**, 377–391
- 35 Scholer, H. R., Ruppert, S., Balling, R., Suzuki, N., Chowdhury, K. and Gruss, P. (1990) New type of POU domain in germ line-specific protein Oct-4. *Nature (London)* **344**, 435–439
- 36 Okazawa, H., Okamoto, K., Ishino, F., Ishino-Kaneko, T., Takeda, S., Toyoda, Y., Muranatsu, M. and Hamada, H. (1991) The oct3 gene, a gene for an embryonic transcription factor, is controlled by a retinoic acid repressible enhancer. *EMBO J.* **10**, 2997–3005
- 37 Scott, M. P. (1992) Vertebrate homeobox gene nomenclature. *Cell (Cambridge, Mass.)* **71**, 551–553
- 38 Shimazaki, T., Okazawa, H., Fujii, H., Ikeda, M., Tamai, K., McKay, R. D., Muramatsu, M. and Hamada, H. (1993) Hybrid cell extinction and re-expression of Oct-3 function correlates with differentiation potential. *EMBO J.* **12**, 4489–4498
- 39 Dailey, L., Yuan, H. and Basilico, C. (1994) Interaction between a novel F9-specific factor and octamer-binding proteins is required for cell-type-restricted activity of the fibroblast growth factor 4 enhancers. *Mol. Cell. Biol.* **14**, 7758–7769
- 40 Yuan, H., Corbi, N., Basilico, C. and Dailey, L. (1995) Developmental-specific activity of the FGF-4 enhancer requires the synergistic action of Sox2 and Oct-3. *Genes Dev.* **9**, 2635–2645
- 41 Liu, L. and Roberts, R. M. (1996) Silencing of the gene for the  $\beta$  sub-unit of human chorionic gonadotropin by the embryonic transcription factor Oct-3/4. *J. Biol. Chem.* **271**, 16683–16689
- 42 Ambrosetti, D. C., Basilico, C. and Dailey, L. (1997) Synergistic activation of the fibroblast growth factor 4 enhancer by Sox2 and Oct-3 depends on protein–protein interactions facilitated by a specific spatial arrangement of factor binding sites. *Mol. Cell. Biol.* **17**, 6321–6329

Received 17 April 2003/9 June 2003; accepted 3 July 2003

Published as BJ Immediate Publication 3 July 2003, DOI 10.1042/BJ20030579