Expression of the FOXP1 Transcription Factor is Post-Transcriptionally Silenced in Normal and Malignant CD138$^+$ Plasma Cells

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Abstract: The FOXP1 transcription factor is heterogeneously expressed in normal B cells and is highly expressed in poor prognosis B-cell lymphoma patients. Double immunohistochemical labelling studies identified the striking absence of FOXP1 protein expression in VS38c$^+$, CD38$^+$ and CD138$^+$ plasma cells; prompting an investigation of FOXP1 mRNA and protein expression in multiple myeloma (MM) and the pre-neoplastic plasma cell proliferation monoclonal gammopathy of undetermined significance (MGUS). *FOXP1* mRNA expression was assessed by quantitative RT-PCR in normal CD138$^+$ bone marrow plasma cells, MM cell lines (n=4) and cases of MM, including aspirates of whole BM (n=11) and purified CD138$^+$ cells (n=12). Surprisingly both normal and abnormal CD138$^+$ plasma cells expressed the *FOXP1* transcript, some cases of each exhibiting high levels, comparable to those in activated B-cell-like diffuse large B-cell lymphoma. However normal CD138$^+$ bone marrow plasma cells, MM cell lines and CD138$^+$ plasma cells in primary MGUS (n=13) and MM biopsies (n=68) were largely devoid of FOXP1 protein expression. The notable exception was two MM patients in which >30% of the CD138$^+$ population was FOXP1$^+$. Mechanisms which block mRNA translation or de-stabilise the FOXP1 protein may silence its expression in plasma cells.

Keywords: FOXP1, plasma cell, multiple myeloma, post-transcriptional silencing.

INTRODUCTION

Forkhead box P1 (FOXP1) is a member of the forkhead box (FOX) family of transcription factors that are defined by a common DNA-binding domain termed the forkhead box or winged helix domain. The *FOXP1* gene maps to a tumour suppressor locus at 3p14.1 and is aberrantly expressed at both the mRNA and protein levels in a variety of carcinomas [1]. In breast cancer patients the loss of FOXP1 protein expression correlates with a poor prognosis, consistent with a role as a tumour suppressor [2]. In contrast, the high level expression of the FOXP1 protein in large B-cell Non Hodgkin's lymphomas (NHL) correlates with a poor prognosis [3-5]. While this might initially appear to be counter intuitive there are plenty of examples in the published literature of transcription factors, such as Maf [6] and NF-κB [7], that can display dual roles as either tumour suppressors or oncogenes, depending on the cellular context. Certainly the alterations in apoptosis and cellular proliferation observed in cardiac tissues from *Foxp1* knockout mice have indicated that the affects on these processes are regulated both temporally and spatially during development, suggesting that there is complex context dependent regulation of Foxp1 function *in vivo* [8].

The identification of recurrent chromosome translocations targeting the *FOXP1* gene in both mucosal associated lymphoid tissue (MALT) lymphomas and in diffuse large B-cell lymphomas (DLBCL) suggested that FOXP1 de-regulation might have an important role in lymphomagenesis [9-11]. Interestingly these translocations commonly target the coding region of the *FOXP1* gene [12]. This is consistent with our data suggesting that N-terminal truncation of FOXP1 (by alternative splicing) may be a key event in normal B-cell activation that is retained in the activated B-cell-like (ABC) DLBCL subtype [13]. Furthermore, N-terminal truncation of foxP1 is an oncogenic event in a retroviral insertion model identifying novel oncogenes that cause avian nephroblastoma [14]. This raises the possibility that constitutive expression of smaller FOXP1 isoforms may have an oncogenic role in ABC-like DLBCL.

The majority of studies have focussed on FOXP1 in malignancy and thus there is still relatively little information concerning the roles of FOXP1 in normal tissues. Studies using knock out mice have identified an essential role for Foxp1 in embryonic development with phenotypes affecting cardiac [8], neuronal [15, 16], lung [17] and B-cell development [18]. Foxp1 is also required for monocyte differentiation and macrophage function [19, 20], adipocyte differentiation and is implicated in human heart failure. Recently *FOXP1* silencing in *ex vivo* expanded bone marrow mesenchymal stem cells has been reported to suppress their self-renewal capacity and their adipogenic differentiation [21].

A brief description of FOXP1 expression in B cells in reactive tonsil has already reported that this protein is expressed in the majority of mantle zone B cells, a variable proportion of germinal centre (GC) cells and in many cells in

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the interfollicular area [1]. In addition to staining haematopoietic cells, FOXP1 expression was also observed in both tonsillar epithelium and endothelium. In the current study we have employed double immunoenzymatic labelling techniques to identify the absence of FOXP1 protein expression in normal $CD138^+$ tonsillar plasma cells. Despite the presence of *FOXP1* transcripts, the vast majority of both normal and abnormal bone marrow plasma cells also lacked FOXP1 protein expression. Silencing of FOXP1 protein expression may have an important biological role during terminal B-cell differentiation to plasma cells and represents a novel mechanism by which FOXP1 expression is regulated.

METHODS

Patients and Tissues

Patients' bone marrow samples and normal reactive tonsils from routine tonsillectomy were obtained with informed consent from the John Radcliffe Hospital, Oxford and the Dubrava University hospital, Zagreb, Croatia. This study was conducted under ethical approval from the Oxfordshire Clinical Research Ethics Committee.

Immunohistochemistry

Four micron sections of FFPE tonsil or cell pellets were captured on charged slides and dried at 37°C over night. Sections were dewaxed then antigen retrieved by microwaving in 50mM Tris/2mM EDTA, pH 9.0 [1]. The anti-FOXP1 monoclonal antibody, JC12 (in house hybridoma supernatant, 1/80 dilution) was applied for 30 minutes at room temperature and binding was detected using the Envision-HRP Kit and DAB+ substrate (DakoCytomation). For single labelling, sections were counterstained with hematoxylin (Gill's No. 2; Sigma-Aldrich) and mounted in Aquatex (VWR International). For double immunoenzymatic labelling, similar sequential rounds of staining were conducted with DAB+ substrate used in the first round and Vector SG substrate (Vector Laboratories) in the second, without the nuclear counterstain. The second primary antibodies were as follows: VS38c, undiluted monoclonal hybridoma supernatant; CD138, clone MI15, diluted 1/100 (DakoCytomation); CD38, clone SPC32, diluted 1/100 (Novocastra). After detection, slides were washed in water, dehydrated in xylene and mounted in VectaMount Permanent Mounting Medium (Vector Laboratories).

Cell Lines

MM cell lines included in the study were JJN3, NCIH929, RPMI8226 and THIEL. DLBCL cell lines OCI-Ly3, OCI-Ly10 (ABC-derived), SUDHL6, SUDHL10 and DB (GC-derived) were a kind gift from Dr Eric Davis (National Cancer Institute, Bethesda, MD), RIVA (ABC-derived) was a kind gift from Professor Martin Dyer (Leicester University, United Kingdom) and HLY-1 (ABC-derived) was generously provided by Dr Talal Al Saati (Purpan Hospital, Toulouse, France). Cell lines were maintained in RPMI 1640 media supplemented with 10% FCS, 2mM glutamine and antibiotics [streptomycin (50µg/ml) and penicillin (50U/ml)] at 37°C and 5% CO_2. For each line, approximately $1x10^7$ cells were formalin-fixed and paraffin embedded, additional cell pellets were snap frozen and used for Western blotting or RNA extraction.

qRT-PCR

$CD138^+$ cells were isolated from bone marrow (BM) aspirates using MACS microbeads, according to the manufacturer's instructions (Miltenyi Biotec). Total RNA was extracted from $CD138^+$ cells and cell lines using the RNeasy Mini Kit (Qiagen). 100ng of total RNA was reverse transcribed using random primers (Promega) and Superscript III reverse transcriptase (Invitrogen). RNA from BM aspirates was isolated using QIAamp RNA Blood Mini Kit (Qiagen) and one microgram of total RNA was used for cDNA synthesis using GeneAmp Gold RNA PCR Core Kit (Applied BioSystems). 20 µl multiplex qPCR reactions were setup in triplicate using 1x EXPRESS qPCR SuperMix with ROX reference dye (Invitrogen), 1µl FOXP1-FAM TaqMan probe (Hs00212860_m1), 1µl TBP-VIC TaqMan probe (*TBP*; 4326322E) (Applied Biosystems), 1µl GAPDH-CY5 custom probe (150 nM primers, 250nM probe, final concentration) (Eurogentec) and 1 µl of cDNA template. Real-Time PCR amplification was performed in triplicate with a Chromo 4 continuous fluorescence detector (MJ Research). *FOXP1* mRNA expression is presented as relative to that in the DB DLBCL cell line which was assigned a value of 1.

Western Blotting

Nuclear extracts were prepared from approximately $1x10^7$ cells using the Nuclear Extraction Kit (Panomics). Extracts were resolved on 10% SDSPAGE gels and transferred to Hybond nitrocellulose membrane (GE Healthcare) using semidry apparatus. Membranes were blocked for 1 hour with PBS/5% dried skimmed milk powder, 0.02% Tween. The JC12 antibody was used to probe the membrane at 1/10 dilution in blocking buffer on a rocking platform at 4°C overnight. The membrane was washed (3 x 10 minutes) in PBS/0.05% Tween, then the secondary antibody, goat anti-mouse Ig-HRP (DakoCytomation) was applied at a 1/5000 dilution in blocking buffer for 1 hour at room temperature. The wash steps were repeated and the membrane was developed using enhanced chemiluminescence (ECL) reagent (GE Healthcare). The membrane was subsequently reprobed with the anti-TBP antibody (clone 1TBP18, Abcam) used at 1/1000 and detected as above to confirm adequate sample loading.

RESULTS

Using double immunoenzymatic labelling studies we have identified the absence of FOXP1 protein expression in terminally differentiated $CD38^+$, $CD138^+$ or $VS38c^+$ plasma cells from tonsil (Fig. **1**). The occasional $CD38^+/FOXP1^+$ and $CD138^+/FOXP1^+$ lymphocytes were noted, indicating that a relatively rare population of terminally differentiated B cells do express FOXP1.

While analysing FOXP1 expression in lymphoma cell lines we were surprised to find that the JJN3 MM cell line expressed relatively high levels of the *FOXP1* mRNA (Fig. **2**). The levels were comparable to those in the HLY1 and DB DLBCL cell lines, which we have previously shown to strongly express the FOXP1 mRNA and protein [13]. The

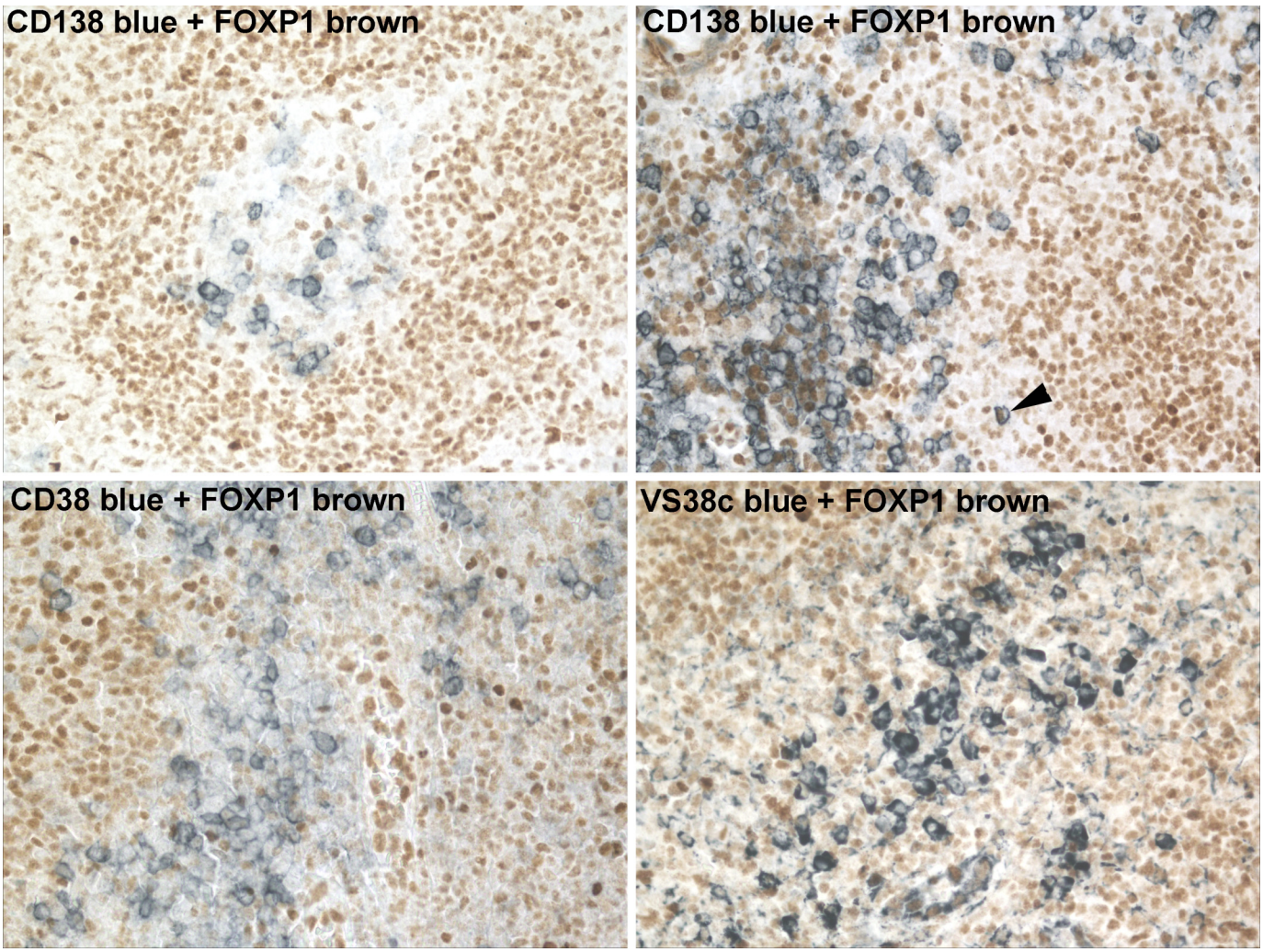

Fig. (1). Double immunoenzymatic labelling of formalin-fixed paraffin embedded tonsil sections. FOXP1 (brown) is rarely co-expressed with markers of terminally differentiated B cells (CD138, CD38, VS38c; blue). The black arrow head in the top righthand panel illustrates a rare plasma cell that is double labelled with CD138 and FOXP1.

NCIH929 and RPMI8226 cell lines expressed low levels of *FOXP1* mRNA, while none was detectable in the THIEL cell line. Western blotting studies showed that FOXP1 protein expression was barely detectable in the majority of MM cell lines, in contrast to the highly FOXP1-positive OCI-Ly3 and DB DLBCL cell lines used as controls (Fig. **3**). Interestingly the JJN3 cell line weakly expressed both the full-length FOXP1 protein and similar levels of the two smaller isoforms that are also present in the OCI-Ly3 DLBCL cell line. Using immunoenzymatic labelling to double stain the MM cell lines for FOXP1 expression (brown) and CD138 (blue) we observed no FOXP1 protein expression in the NCIH929 and THIEL cell lines, while the occasional FOXP1 positive nucleus was detected in the RPMI8226 and JJN3 cell lines.

This study was extended to investigate, whether abnormal and malignant plasma cells in bone marrow also lacked FOXP1 expression. In whole bone marrow aspirates from Croatian patients without plasma cell malignancy or those with MGUS or MM there was no significant difference in *FOXP1* mRNA levels between patient groups (Fig. **4a**). Although, most samples expressed relatively high levels of *FOXP1* mRNA there was no correlation with the expression levels of *CD138* mRNA, which was used to assess the level of plasma cell infiltration (data not shown; Campbell *et al*., Br J Haem 2010; in press).

Immunolabelling studies subsequently identified the widespread expression of the FOXP1 protein in CD138-negative bone marrow cells (Fig. **5**). Therefore, *FOXP1* mRNA expression was also further investigated in $CD138^+$ purified bone marrow plasma cells from a cohort of patients from Oxford to exclude other cell populations from the expression analysis (Fig. **4b**). *FOXP1* was differentially expressed between the $CD138^+$ bone marrow samples, but there was no evidence for elevated *FOXP1* mRNA expression in malignant plasma cells when compared to those from MGUS patients or patients without plasma cell malignancy. However *FOXP1* mRNA was detectable in all the plasma cell samples, a significant proportion of which (26/30, 86.6%) expressed levels comparable to that seen in some strongly positive DLBCL cell lines, such as DB and the ABC-DLBCL lines OCI-Ly3 and OCI-Ly10 [13]. Interestingly, the levels of *FOXP1* mRNA in both normal and abnormal $CD138^+$ bone marrow plasma cells was

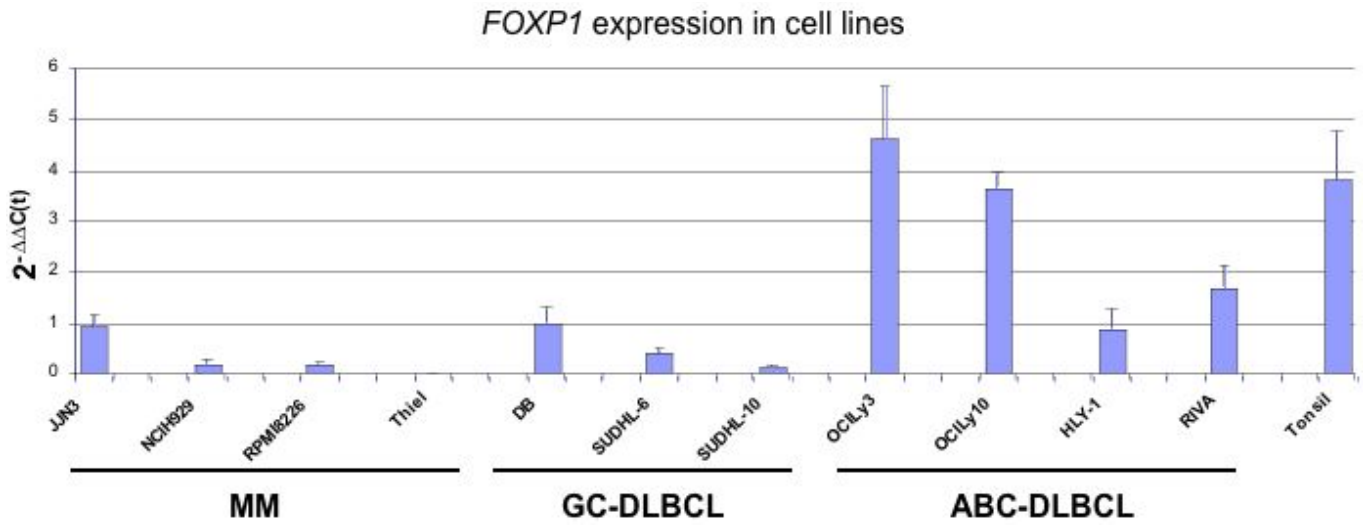

Fig. (2). *FOXP1* relative mRNA expression in MM and DLBCL-derived cell lines. Highest expression levels are observed in ABC-like DLBCL, although the GC-DLBCL line DB and the MM line JJN3 also express significant levels of *FOXP1* transcripts.

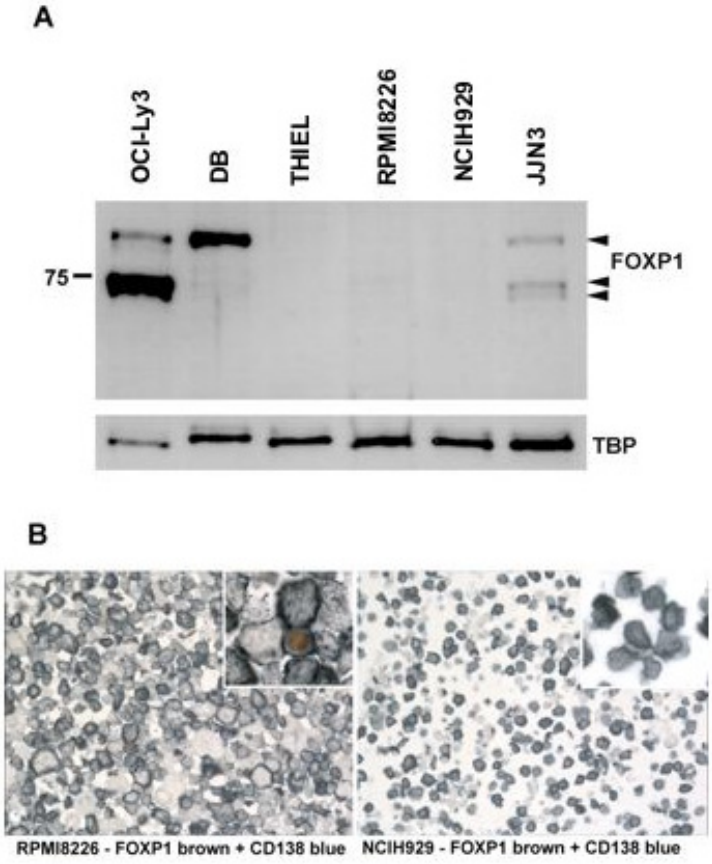

Fig. (3). A. Western blotting of nuclear extracts from DLBCL and MM-derived cell lines. Probing with the anti-FOXP1 monoclonal antibody JC12 shows a lack of FOXP1 protein expression in MM as compared with DLBCL samples. Weak expression of three FOXP1 protein isoforms was detectable in the JJN3 cell line. TBP was used to indicate sample loading. B. Double immunoenzymatic labelling of MM-derived cell lines; FOXP1 staining in brown and CD138 in blue. The Presence of occasional $FOXP1^{+}CD138^{+}$ double labelled cells was detected in RPMI8226 cells, whereas no FOXP1 labelling was detectable in the NCIH929 cell line.

generally higher than those observed in the MM-derived cell lines. These data suggest that *FOXP1* mRNA expression is not commonly transcriptionally silenced in normal or abnormal plasma cells.

Double immunoenzymatic labelling was used to investigate the expression of FOXP1 at the protein level in $CD138^{+}$ plasma cells. Paraffin embedded bone marrow trephines were available from fifteen Croatian patients (including nine MM patients and two MGUS patients) of

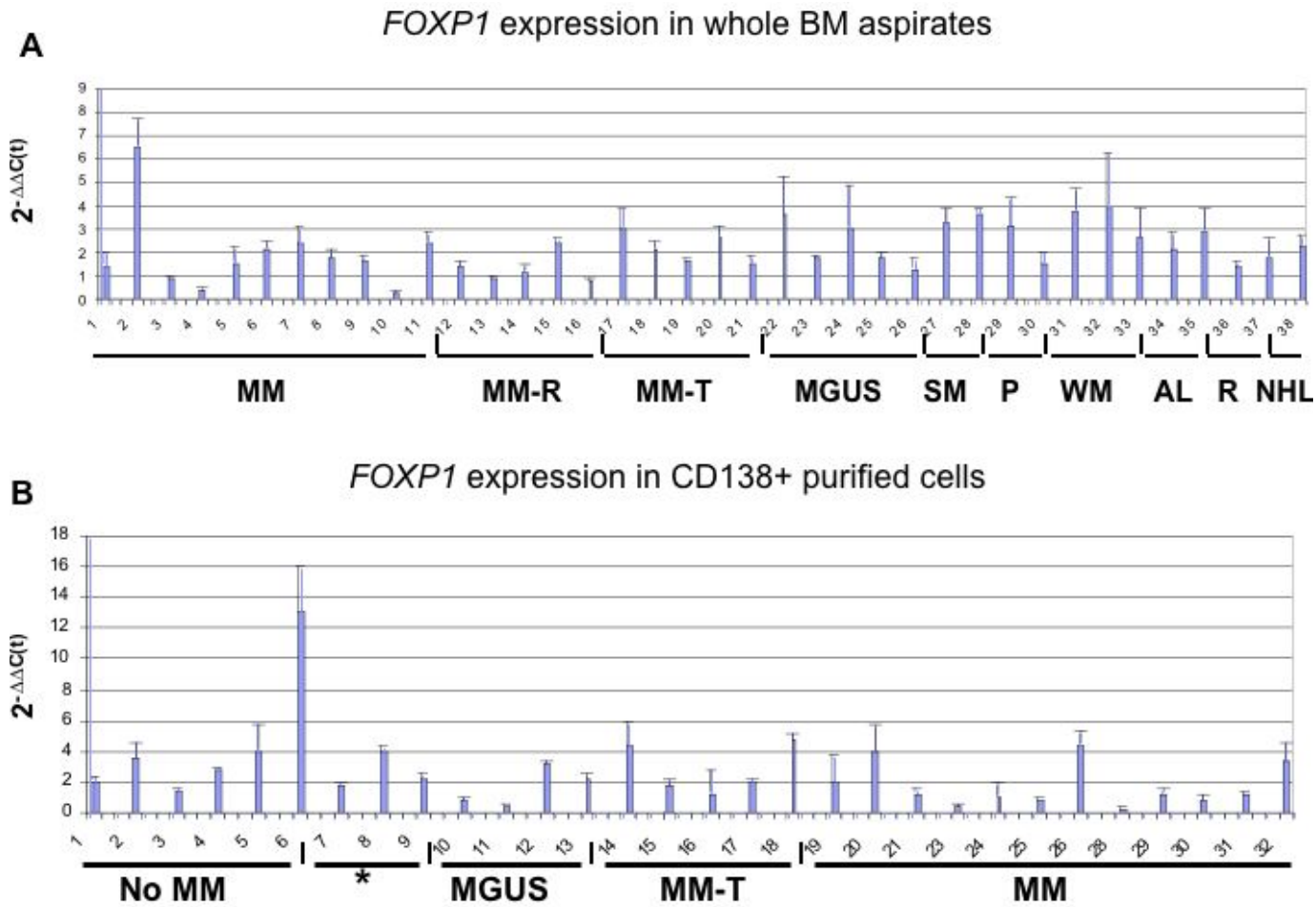

Fig. (4). *FOXP1* relative mRNA expression in (**A**) total bone marrow aspirates and (**B**) purified CD138$^+$ plasma cells. MM = multiple myeloma at diagnosis; MM-R = MM patients at relapse; MM-T, refers to samples taken from patients post-treatment; SM = smoldering myeloma; P = plasmacytoma; WM = Waldenstroms Macroglobulinaemia; AL = amyloid; R = reactive marrow; NHL= non-Hodgkin's lymphoma; no MM, includes a heterogenous group of patients whose plasma cells should not be malignant or pre-malignant. * These patients did not fall into one of the above categories. Bone marrow trephines from cases 7 and 8 were reported as LPL or myeloma, clinically the diagnosis was LPL; case 9 had a bone plasmacytoma, the bone marrow aspirate was not reported to show increased plasma cells and the trephine was inadequate.

which ten (patients numbered; 1, 2, 3, 10, 12, 22, 23, 27, 31 and 32) corresponded to those where the expression levels of *FOXP1* mRNA had been determined in whole bone marrow. There was no FOXP1 protein detected in fourteen of the patients' biopsies. Samples with occasional rare FOXP1$^+$ plasma cells were scored as negative throughout this study. The exception was patient number 3, where weak/moderate expression of the FOXP1 protein was observed in the nuclei of the majority of the CD138$^+$ population (Fig. *5*, bottom right). This 61 year old male patient was diagnosed with multiple myeloma, IgG kappa paraprotein of 63g/l, albumin 22g/l, Beta2microglobulin 7.0, (stage 3 ISS), with >80% plasma cell infiltrate in the aspirate/trephine and had multiple osteolytic lesions. He initially received VAD and thalidomide that had to be discontinued due to the cardiac side effects (bradycardia, arrhythmia). He was continued on Dexamethasone with which he achieved partial remission. The patient received tandem autologous stem cell transplants and is still alive two years after diagnosis. An unusual feature of this patient was that the diagnostic bone marrow sample was taken while he was recovering from sepsis (*Streptococcus pneumoniae)*. We cannot exclude the possibility that this event may have affected the expression of FOXP1; certainly cytokines such as IL-2, IL-10 and TGF-beta have a key role in the development and function of regulatory T-cells characterised by the expression of the related FOXP3 protein. However, further studies will be needed to determine whether cytokines do indeed also have a role in modulating FOXP1 expression and function. Interestingly, this case had one of the lowest levels of *FOXP1* mRNA expression suggesting that *FOXP1* mRNA levels do not reflect tumoural FOXP1 protein expression in whole bone marrow aspirates.

FOXP1 protein expression was also investigated by double immunoenzymatic labelling in a larger series of patients from Oxford. Including routinely fixed bone marrow trephines from non-malignant reactive marrows (n=10), patients with MGUS (n=11) and a cohort with MM (n=60). The vast majority of normal and malignant bone marrow CD138$^+$ plasma cells lacked FOXP1 protein expression. The presence of CD138-negative cells expressing FOXP1 served as an internal control for the immunolabelling technique.

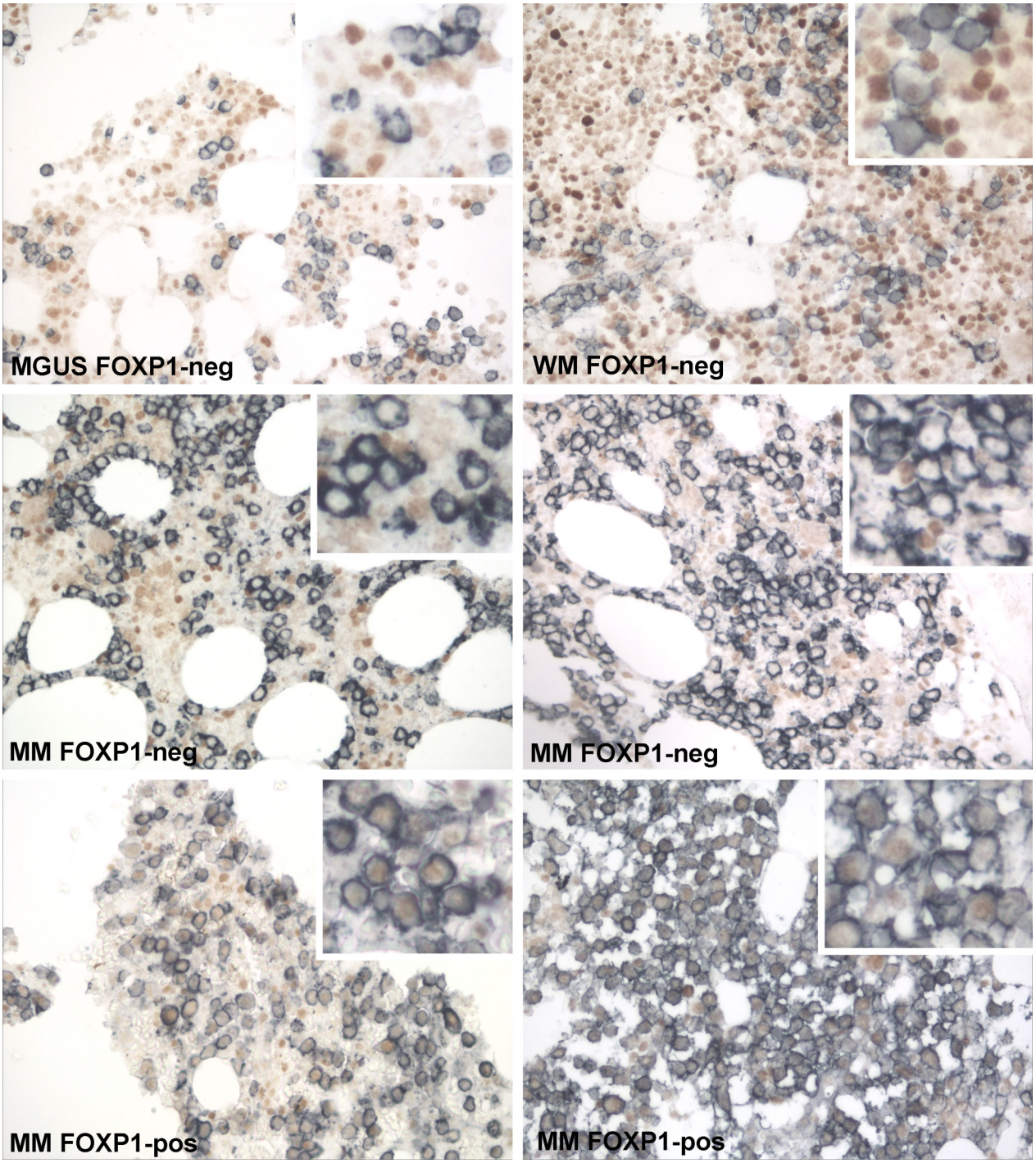

Fig. (5). Double immunoenzymatic labelling of bone marrow samples; FOXP1 brown and CD138 blue. As indicated by the top four panels the majority of pre-malignant and malignant $CD138^+$ plasma cells lacked expression of the FOXP1 protein, although there were many $CD138^-FOXP1^+$ cells in this tissue. The detection of >30% $FOXP1^+/CD138^+$ cells was restricted to two MM cases, one from Oxford (bottom left) and the other from Zagreb (bottom right).

FOXP1 protein expression in more than 30% of $CD138^+$ cells was only observed in one MM biopsy, taken at time of diagnosis (Fig. **5**, bottom left). The patient had fairly typical clinical features including: age 69 years at diagnosis, 90% plasma cell infiltrate on trephine, no bone marrow involvement, normal calcium, anaemic at diagnosis with haemaglobin of 8.7, normal renal function, IgA lambda paraprotein, albumin 37, Beta2microglobulin 10.8 and was still alive after 7 months follow up. A minor population of $FOXP1^+/CD138^+$ plasma cells (<10%) was also observed in two MM cases and one MGUS case. These data suggest that FOXP1 is not frequently expressed at the protein level in MM.

DISCUSSION

Foxp1 has been shown to function during the pro-B to pre-B cell transition in the early stages of B-cell development (Hu *et al*., 2006) and regulates *Rag* gene

expression. Our immunolabelling studies have already shown widespread, high level FOXP1 expression in lymphoid tissue, in particular in the naïve B cells of the mantle zone and variable but often strong expression in B cells within the germinal centre [1]. However, here we show that expression of the FOXP1 protein appears to be tightly regulated in the terminal stages of B-cell development, rarely being expressed in normal plasma cells. However, *FOXP1* transcripts were expressed at relatively high levels in $CD138^+$ bone marrow plasma cells from patients without MM.

This pattern of FOXP1 protein expression, with occasional nuclear positivity, was found to be similar in MM cell lines and *FOXP1* transcripts were fairly abundantly expressed in the JJN3 myeloma cell line. In the two MM cell lines in which FOXP1 protein was detectable, it appeared that this was restricted to moderate levels of nuclear expression in a very minor population of $CD138^+$ cells. Similarly, we observed that the FOXP1 protein is rarely expressed in malignant plasma cells, yet the *FOXP1* transcript is abundant in samples of purified $CD138^+$ cells recovered from MM and MGUS BM aspirates. It was noticeable that both normal and malignant $CD138^+$ primary bone marrow cells tended to expressed considerably higher levels of the *FOXP1* mRNA than was observed in three of the four MM-derived cell lines. It is possible that factors in the bone marrow microenvironment may upregulate *FOXP1* mRNA expression in bone marrow plasma cells. Certainly expression of the related FOXP3 transcription factor in regulatory T cells is significantly affected by cytokines, such as IL-2 and the signalling molecule TGF-β [22]. These data suggest that the expression of the FOXP1 protein in both normal and abnormal plasma cells is regulated at a post-transcriptional level.

While we were preparing this manuscript, another group published a paper and a case report describing FOXP1 protein expression in MM; *FOXP1* mRNA expression was not analysed in either study [23]. The case report described a $FOXP1^+$ MM case with rather atypical clinical features, hyperploidy and multiple *IGH* translocations in which the *FOXP1* gene was amplified in $CD138^+$ cells (12-15 copies) [23]. In a larger series of 13 MGUS and 60 MM patients they identified >30% nuclear FOXP1 protein expression in one MGUS and 12 MM biopsies (20%) [24]. Korac *et al.*, detected FOXP1 protein expression at a much higher incidence than in our study, as we detected >30% FOXP1 nuclear positivity in only 3% of MM patients (2/68) and none of the MGUS patients (n=13).

The reason(s) for the difference in the findings are unclear. Both groups have used the same monoclonal anti-FOXP1 antibody to detect protein expression in formalin-fixed paraffin-embedded bone marrow trephines by immunohistochemistry (although different labelling kits were used). There was nothing to suggest that FOXP1 expression differed between the cases that we studied from Oxford versus those from Croatia. However, we found that double labelling enabled us to detect weak FOXP1 protein expression levels that would have been difficult to detect in the presence of a nuclear counterstain and to verify that the positive cells were indeed $CD138^+$ plasma cells. As illustrated by the data presented in Fig. (**5**) we observed variable and often significant levels of FOXP1 expression in the CD138-negative population in bone marrow.

Korac and colleagues identified *FOXP1* copy number changes in 24/60 MM and 5/13 MGUS [24]. How this relates to the expression of the FOXP1 protein is unclear as the abnormalities were detected at a much higher frequency than was FOXP1 protein expression. The authors did not comment on whether the MM cases reported to express the FOXP1 protein included those with the highest *FOXP1* gene copy number. Further study will be necessary to determine whether these genetic changes genuinely contribute to aberrant FOXP1 protein expression in MM. Certainly our data suggest that the *FOXP1* mRNA is commonly expressed in both normal and malignant plasma cells in the absence of FOXP1 protein expression.

The rarity of FOXP1 protein expression in normal plasma cells is consistent with the detection of FOXP1 protein expression in only a minority of MM. Other transcription factors with a role in early B-cell development are also involved in terminal differentiation to plasma cells, including PAX5 which is silenced during plasma cell development and IRF4 which is up-regulated during plasma cell differentiation. Thus it is not without precedent to suggest that the absence of FOXP1 protein expression may also be functionally important during terminal B-cell differentiation to plasma cells. It will be interesting in the future to investigate the affects that expressing FOXP1 has on the terminally differentiated phenotype of plasma cells. In particular whether FOXP1 regulates the expression of those cell surface markers and transcription factors whose silencing or induction plays a crucial role in this phenotype. The post-transcriptional regulation of FOXP1 protein expression in both normal and malignant plasma cells provides an explanation as to why its loss of expression would not have been detected by studies of gene expression changes occuring during plasmacytic differentiation. *FOXP1* mRNA levels are indicative of the FOXP1 protein expression patterns in lymphomas derived from mature B cells. Therefore, it will be interesting to discover the mechanism of this novel post-transcriptional regulation of FOXP1 expression in terminally differentiated B cells.

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