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Regulation of the *FABP7* gene by PAX6 in malignant glioma cells

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ABSTRACT

Brain fatty acid-binding protein (FABP7) and PAX6 are both expressed in radial glial cells and have been implicated in neurogenesis and glial cell differentiation. FABP7 and PAX6 have also been postulated to play a role in malignant glioma cell growth and invasion. Here, we address the role of PAX6 in regulating *FABP7* gene expression in malignant glioma cells. We report that *PAX6* and *FABP7* RNA are generally co-expressed in malignant glioma cell lines, tumors and tumor neurospheres. Using the CAT reporter gene assay, we show that *FABP7* promoter activity is upregulated by PAX6. Sequential deletion analysis of the *FABP7* promoter, combined with gel shift and supershift assays demonstrate the presence of a PAX6 responsive region located upstream of the *FABP7* gene, at −862 to −1033 bp. Inclusion of sequences between −1.2 and −1.8 kb reduced CAT activity, suggesting the presence of a repressor element within this region. While PAX6 overexpression did not induce endogenous FABP7 expression in FABP7-negative cells, knock-down of PAX6 in PAX6-positive malignant glioma cells resulted in reduced FABP7 levels. These data provide the first evidence of direct transactivation of the *FABP7* proximal promoter by PAX6 and suggest a synergistic mechanism for PAX6 and other co-factor(s) in regulating *FABP7* expression in malignant glioma.

1. Introduction

Grades III and IV astrocytomas, collectively called malignant glioma, are highly infiltrative tumors with glial cell properties. FABP7 [a.k.a brain fatty acid-binding protein (B-FABP) or brain lipid-binding protein (BLBP)], is an intracellular lipid binding protein expressed in radial glial cells during brain development [1,2]. FABP7 is also expressed in a subset of malignant glioma cell lines [3,4]. Expression of FABP7 in malignant glioma cells that do not normally express FABP7 leads to increased cell migration, whereas reduction of FABP7 in malignant glioma cells that express FABP7 results in decreased cell migration [4,5]. Of note, expression and nuclear distribution of FABP7 in grade IV astrocytoma tumors negatively correlates with patient survival [6,7].

PAX6, a transcription factor that belongs to the paired box family, is highly conserved among vertebrates. PAX6 is expressed in the developing eye where it plays an essential role in morphogenesis [8,9]. PAX6 is also expressed in the developing forebrain, hindbrain and spinal cord where it has been shown to play roles in neurogenesis, astrocyte differentiation and self-renewal of radial glial cells [10–13]. The spatiotemporal expression pattern of PAX6 in developing rat brain is similar to that of FABP7 [11]. Cortical neuroepithelial cells and radial glial cells, specifically shown to express FABP7, also express PAX6 [14]. Like FABP7, PAX6 controls the migration of neuronal cell precursors along radial glia processes in the cortical subventricular zone [1,15,16]. Importantly, mutation of *Pax6* in rats (*rSey*^{2}/*rSey*^{2}) leads to downregulation of the *Fabp7* gene in embryonic brain [11,17].

PAX6 and FABP7 are both expressed in human astrocytoma tumor tissues and malignant glioma cell lines derived from these tumors [3,4,18,19]. To determine whether PAX6 can directly regulate *FABP7* transcription in malignant glioma cells, we tested the effect of PAX6 expression on a *FABP7* promoter-driven reporter gene. We show that the *FABP7* promoter is bound and transactivated by PAX6 and that reduction in endogenous PAX6 levels results in decreased FABP7 expression.

2. Materials and methods

2.1. Cell lines, DNA constructs and cell culture conditions

The source and culture conditions for the human malignant glioma cell lines employed in this study have been previously described [3,20,21]. The neurosphere cultures generated from grade IV astrocytomas were provided by Drs. Hua Chen and Kenneth Petruk (University of Alberta). pSG5-PAX6 was obtained from Dr. Simon Saule (Institut Curie Section Recherche, France).

Abbreviations: FABP, fatty acid-binding protein; PAX6, paired box protein 6; CAT, chloramphenicol acetyl transferase; siRNA, small interfering RNA.

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pCAT constructs containing different amounts of 5′-flanking DNA (1.8, 1.2, 1 kb, 660, 400, 240 and 140 bp) have been previously described [21].

2.2. RT-PCR

Total RNA was isolated from transfected and non-transfected human malignant glioma cell lines using TRIzol reagent (Invitrogen). Reverse transcription and PCR conditions have been described previously [22]. The following oligonucleotide primers were used for PCR amplification: *PAX6* sense 5′-GAGCCAGCATGCAGAACAG-3′, antisense 5′-TGCCCGTTGAACATCCTTAG-3′; *FABP7* and actin primers have been previously described [22].

2.3. Northern Blot analysis

Conditions for RNA isolation and Northern Blotting have been previously described [3]. The following probes were used: 450 bp *Eco*RI/*Pst*I cDNA fragment from human *FABP7*, 400 bp *Hin*dIII/*Eco*RV cDNA fragment from human *PAX6* cDNA and 500 bp *Bam*HI cDNA fragment from human β-actin.

2.4. Western Blot analysis

Nuclear and cytoplasmic protein extracts were prepared according to the protocol of Dignam et al. [23]. Proteins were electrophoresed in 15% polyacrylamide–SDS gels and electroblotted onto nitrocellulose membranes. Blots were immunostained with mouse anti-PAX6 antibody (Hybridoma Bank, University of Iowa; 1:1000), rabbit anti-human FABP7 antibody (1:1000) [13] and mouse anti-human β-actin antibody (Sigma; 1:50,000).

2.5. Electrophoretic mobility shift assay

Gel shifts were carried out as previously described [24] using DNA fragments spanning −133 to −513 bp, −654 to −1033 bp, −654 to −878 bp, −862 to −972 bp, −920 to −1133 bp, relative to the human *FABP7* transcription start site. For the supershift assay, 1 μL of anti-PAX6 antibody (Hybridoma Bank, University of Iowa) was added to the binding reaction 10 min after addition of the DNA probe. DNA–protein complexes were resolved in a 5% or 6% polyacrylamide gel in 0.5× TBE for 1.5–3 h.

2.6. PAX6 overexpression and CAT assay

Five micrograms of either pSG5-PAX6 expression construct or pSG5 empty vector were co-transfected with 5 μg of one of the following pCAT constructs: −1785 to +20 bp with +1 indicating the transcription start site of *FABP7*; −1.2 kb to +20; −1.0 kb to +20; −660 to +20 bp; −400 to +20 bp; −240 to +20 bp; −140 to +20 bp). Two to four independent experiments were carried out for each CAT construct tested. The CAT assay procedure has been described previously [21].

2.7. siRNA transfection

The following Stealth siRNAs (Invitrogen) were used to knock down PAX6: 5′-CGUGUCCAACGGAUGUGUGAGUAAA-3′ and 5′-GAAAGAGUUUGAGAGAACCCAUUAU-3′; or PBX1: 5′- CCACAGAAU GAAGCCUGCCUUGUUU-3′ and 5′AAACAAGGCAGGCUUCAUUCUG UGG-3′. M103 malignant glioma cells were transfected with either 10 nM PAX6 siRNA or 10 nM control siRNA using RNAi-MAX Lipofectamine reagent (Invitrogen). After 24 h, the medium was replaced with DMEM containing 10% FCS and the cells cultured for an additional 60 h.

3. Results

3.1. Expression of PAX6 and FABP7 in human malignant glioma cell lines and tumor biopsies

To investigate a possible relationship between PAX6 and FABP7 in human malignant glioma cells, we examined *PAX6* and *FABP7* mRNA levels in 10 malignant glioma cell lines, half of which express FABP7. Based on Northern Blot analysis, *PAX6* mRNA is found in a subset of malignant glioma lines, with highest levels of *PAX6* and *FABP7* observed in M103 (Fig. 1A). Next, we examined *PAX6* and *FABP7* co-expression in seven brain tumor biopsies: five grade IV astrocytomas, one grade III astrocytoma and one oligodendroglioma. All seven tumors express *PAX6* and *FABP7* RNA (Fig. 1B). There was a positive correlation between *PAX6* and *FABP7* transcript levels, with the exception of one grade IV astrocytoma (lane 6).

PAX6 and *FABP7* are normally expressed in radial glial cells which have neural stem/progenitor cell properties [25]. We therefore examined whether *PAX6* and *FABP7* are also expressed in malignant glioma neurosphere cultures previously shown to have

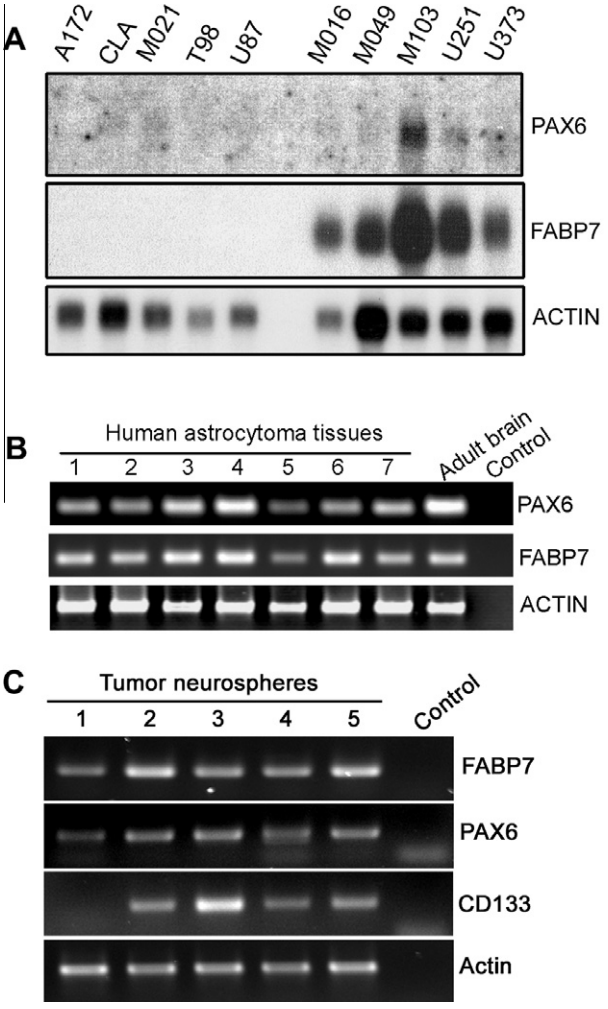

Fig. 1. *PAX6* and *FABP7* RNA in human malignant glioma cells and astrocytoma tumor biopsies. (A) Northern Blot analysis of *PAX6* and *FABP7* in five FABP7-negative (A172, CLA, M021, T98, U87) and five FABP7-positive (M016, M049, M103, U251, U373) malignant glioma cell lines. (B) RT-PCR analysis of *PAX6* and *FABP7* in seven human glioma tumor biopsies: samples 1, 2, 3, 5, 6 are from grade IV astrocytomas; sample 4 is from an oligodendroglioma, sample 7 is from a grade III astrocytoma. Normal adult brain tissue served as the positive control. (C) RT-PCR analysis of *PAX6* and *FABP7* in five neurosphere cultures derived from malignant glioma tumors. Control reactions contain all reagents except for the cDNA template.

characteristics of neural stem cells [26]. *PAX6* and *FABP7* RNA were both found in all five neurosphere cultures tested. CD133, a marker of tumor initiating cells, was expressed in 4 of the 5 neurosphere cultures (Fig. 1C).

3.2. Transactivation of the FABP7 promoter by PAX6

To examine the effect of PAX6 on the *FABP7* promoter, we generated a series of *FABP7* promoter deletions, from −1.8 kb to −140 bp using the *FABP7* transcription start site as the reference point (Fig. 2A) [21]. These promoter fragments were inserted upstream of the chloramphenicol acetyl transferase (CAT) reporter gene. As shown in Fig. 2B, similar levels of CAT activity were obtained with the −140 and −240 bp constructs, whether PAX6 was present or not. A ~1.2–2× increase in CAT activity was observed with the −400 and −660 bp constructs, and a >4× increase in CAT activity was observed with the −1.0 and −1.2 kb constructs in the presence of PAX6. The addition of an extra 600 bp (to −1.8 kb) resulted in a 3× decrease in CAT activity relative to the −1.0 and −1.2 kb constructs. These data suggest that potential PAX6 positive regulatory elements are located between −240 and −400 bp and between −660 bp and −1 kb upstream of the *FABP7* gene, with a negative regulatory element located further upstream between −1.2 and −1.8 kb. The expression of PAX6 in extracts prepared from PAX6-transfected cells was confirmed by Western Blot analysis (Fig. 2C; Supplementary Fig. 1).

3.3. PAX6 binds to FABP7 promoter sequences

We used the gel shift assay to determine whether potential PAX6 binding elements were located within the *FABP7* promoter region. DNA fragments from −133 to −513 bp and from −654 to −1033 bp relative to the *FABP7* transcription start site were radiolabelled and incubated with nuclear extracts prepared from U87 cells transfected with either the control vector (pSG5) or the PAX6 expression construct. The banding pattern in the pSG5 and PAX6 lanes was similar using the −133 to −513 bp DNA fragment as the probe (data not shown). However, with the

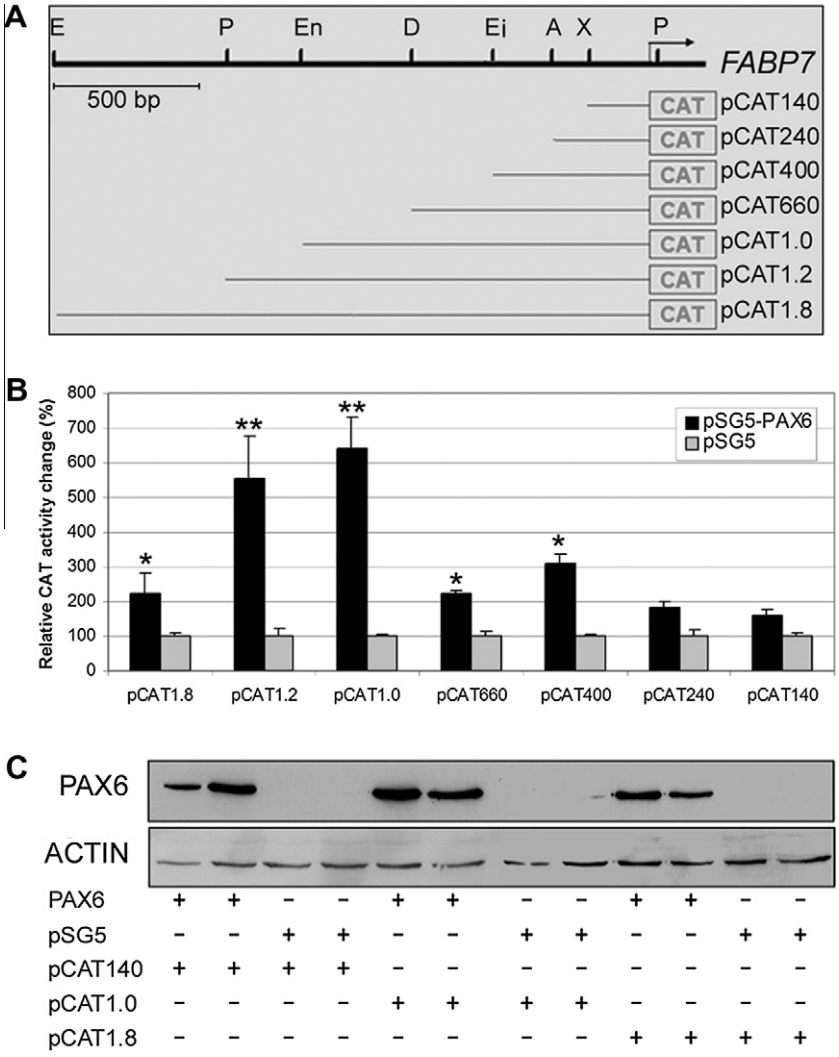

Fig. 2. Transactivation of the *FABP7* promoter by PAX6. (A) Schematic diagram of the *FABP7* promoter showing the transcription start site (arrow) and restriction enzyme sites used for the generation of the pCAT-*FABP7* promoter constructs. Different sizes of 5′ flanking DNA (140, 240, 400, 660 bp, 1, 1.2 and 1.8 kb) were generated by digesting the DNA with *Pst*I (which cuts at +20) and the indicated restriction enzymes (X, *Xho*I; A, *Alu*I; Ei, *Eco*0109I; D, *Dra*I; En, *Eco*NI; P, *Pst*I; E, *Eco*RI. (B) Five micrograms of pCAT constructs (pCAT140, pCAT240, pCAT400, pCAT660, pCAT1.0, pCAT1.2 or pCAT1.8) were co-transfected with 5 μg of either pSG5-PAX6 or empty vector (pSG5) into U87 cells. For each pCAT construct, we report the percent CAT activity obtained with pSG5-PAX6 co-transfection relative to CAT activity obtained with empty vector co-transfection (with the latter set at 100%). Each transfection experiment was carried out two to four times. Results were averaged with standard errors indicated by error bars. Statistical significance is indicated with "∗" ($p < 0.05$) or "∗∗" ($p < 0.01$). (C) Western Blot analysis of PAX6 and actin in CAT assay supernatants prepared from cells co-transfected with PAX6 or pSG5, and pCAT140, pCAT1.0 or pCAT1.8.

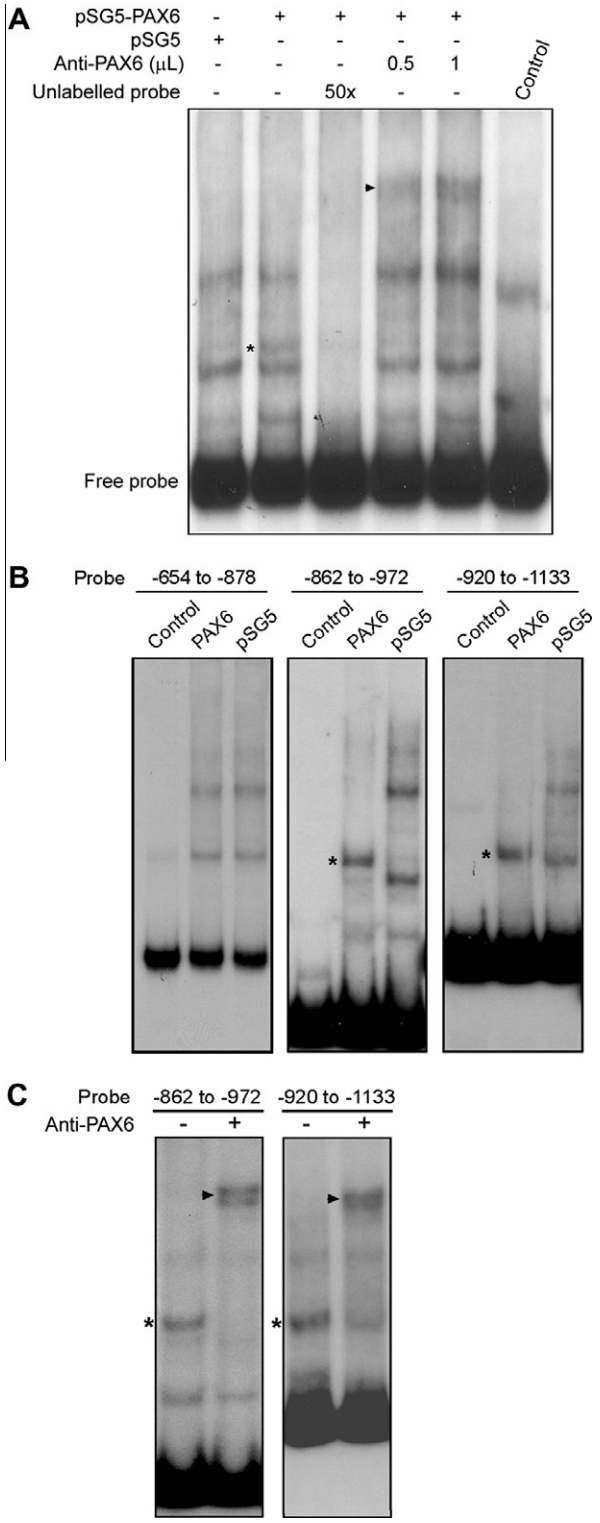

Fig. 3. Binding of PAX6 to *FABP7* promoter. (A) Gel shift assays were carried out with radiolabelled human *FABP7* promoter DNA (−654 to −1033 bp) and nuclear extracts prepared from U87 cells transfected with pSG5 control vector or PAX6 expression construct. Anti-PAX6 antibody (0.5 or 1 μL) was added to the reaction for supershift experiments (lanes 4 and 5). A 50-fold excess unlabeled DNA probe was added to the reaction mixture in lane 3. (B) Gel shift assays were carried out with DNA fragments spanning −654 to −878 bp, −862 to −972 bp and −920 to −1133 bp. Bands specific to PAX6-transfected cells are indicated by the asterisks. Nuclear extracts were omitted in control lanes. (C) Supershift analysis of DNA probes spanning −862 to 972 and −920 to −1133 bp. One microliter of anti-PAX6 antibody was added to the supershift lanes. The PAX6-DNA complexes are indicated by asterisks and the supershifted complexes are indicated by arrowheads.

−654 to −1033 bp DNA fragment as the probe, a band specific to PAX6-transfected cells was identified (Fig. 3A – indicated by asterisk). The signal intensity of all retarded bands was dramatically reduced in the presence of 50× excess unlabeled probe as competitor (lane 3). Addition of anti-PAX6 antibody to the reaction mixture resulted in the disappearance of the PAX6-specific band, with concomitant appearance of a slower migrating band (indicated by the arrowhead).

To further define the PAX6-response region(s) in the *FABP7* promoter, we generated three DNA fragments (spanning −654 to −1133 bp) which served as probes for gel shift assays. A band specific to PAX6-transfected cells was observed with the −862 to −972 bp probe, but not with the −654 to −878 bp probe (Fig. 3B). Incubation of PAX6-positive nuclear extracts with the −920 to −1133 bp probe generated a major band that migrated at a slightly different rate than the lower band observed in PAX6-negative nuclear extracts (Fig. 3B – indicated by asterisk). To investigate whether PAX6 was present in the putative PAX6-specific complexes obtained with probes −862 to −972 bp and −920 to −1133 bp, we performed supershift experiments. Addition of anti-PAX6 antibody resulted in the disappearance of the PAX6-specific band (Fig. 3C – indicated by the asterisks) and the appearance of a slow-migrating band (Fig. 3C – indicated by the arrowheads) for both probes. As the two probes used for these analyses have overlapping regions, these results indicate that the human *FABP7* promoter has at least one upstream PAX6 binding site located between −862 to −1133 bp. As probe −654 to −1033 bp also generated a retarded band that could be supershifted with anti-PAX6 antibody, the region of interest can be further narrowed down to −862 to −1033 bp.

Next, we carried out DNase I footprinting analysis of the −654 to −1262 bp region. A strong well-delineated footprint was observed between −837 and −853 bp; however, this footprint was found in both pSG5 and PAX6 lanes (data not shown). A series of weaker PAX6-specific footprints were detected in the −895 to −1000 bp region, with the most distinct footprints found at −980 to −1000 bp and −903 to −925 bp (Supplementary Fig. 2). The combined supershift and gel shift data suggest that PAX6 may bind to two DNA elements within the −900 to −1000 bp region.

3.4. Effect of PAX6 overexpression on endogenous FABP7 expression

To determine whether ectopic expression of PAX6 can induce endogenous FABP7 expression, we transfected the PAX6 expression construct into the FABP7-negative U87 and T98 malignant glioma cell lines. We then analyzed whether FABP7 transcripts and protein were expressed as a consequence of ectopic PAX6 expression. Neither the *FABP7* mRNA (Fig. 4A) nor FABP7 protein (Fig. 4B) was detected in either the U87 or T98 transfectants, although PAX6 levels were markedly increased in these transfectants. U251 cells which naturally express FABP7 served as a positive control for these experiments (Fig. 4A, B). Transfection of the PAX6 expression construct into FABP7-positive U251 cells had no effect on endogenous FABP7 protein levels (Supplementary Fig. 3). These data indicate that PAX6 by itself cannot induce transcription of *FABP7* in FABP7-negative cells.

3.5. Depletion of PAX6 by siRNA reduces FABP7 expression

To further explore the role of PAX6 in the regulation of *FABP7* transcription, we transfected the PAX6-positive human malignant glioma cell line M103 with PAX6 siRNA. RT-PCR analysis of total RNA prepared from PAX6 siRNA transfectants revealed >90% depletion of *PAX6* transcripts (Fig. 4C). A significant decrease in *FABP7* RNA was observed upon reduction of *PAX6* RNA levels (Fig. 4C).

In comparison, knockdown of *PBX1* in M103 had a minor effect on *FABP7* transcript levels, despite nearly 100% depletion of *PBX1* mRNA (Fig. 4C). PBX1 is a POU-domain transcription factor that has been implicated in the regulation of mouse *Fabp7* expression in the developing brain [27]. In keeping with *FABP7* RNA results, Western Blot analysis showed a >50% reduction in FABP7 protein levels upon PAX6 depletion in M103 cells (Fig. 4D).

4. Discussion

Regulatory factors and pathways associated with *FABP7* expression during brain development may provide important clues regarding *FABP7* regulation in glioma tumors. We have previously demonstrated a role for Nuclear Factor I (NFI) in the modulation of *FABP7* expression in human malignant glioma cells [20,21]. Independent functional studies using *Pax6* or *Fabp7* mutant mice have shown that both these genes affect the neurogenic potential of the cortical radial glia [28,29]. Furthermore, *Pax6* mutation in rat embryonic brains results in a dramatic decrease in *Fabp7* RNA levels, whereas overexpression of exogenous PAX6 in early embryonic brain induces *Fabp7* expression [11]. Together, these data support a role for PAX6 in the regulation of the *Fabp7* gene in developing rat brain. In this report, we demonstrate that PAX6 can directly regulate human *FABP7* transcription. Compelling evidence in support of these data is the observation that knock-down of PAX6 in a FABP7/PAX6-positive malignant glioma cell line results in a significant decrease of FABP7 RNA and protein levels.

Based on promoter deletion analysis, there are both positive and negative PAX6-responsive elements involved in the regulation of the *FABP7* gene. A positive element is located between −660 bp and −1 kb, whereas a predicted negative element is located between −1.2 and −1.8 kb. Our gel shifts and supershift assays demonstrate the presence of a PAX6 responsive region between −862 to −1033 bp relative to the *FABP7* transcription start site, suggesting that the *FABP7* promoter is a direct target of PAX6. DNase I footprints extending from −890 to −1000 kb specifically observed in PAX6-transfected cells support the idea that PAX6 binds to multiple PAX6 recognition sites within this region. Alternatively, PAX6 may interact with one or more transcription factors when bound to this DNA region.

It is clear that regulatory factors in addition to PAX6 regulate *FABP7* transcription in malignant glioma. First, low levels of PAX6 are found in both FABP7-positive and FABP7-negative malignant glioma cell lines. Second, ectopic expression of PAX6 in FABP7-negative malignant glioma cells did not induce endogenous *FABP7* RNA expression. Based on our observation that PAX6 transcription activity is markedly reduced upon inclusion of the distal *FABP7* promoter region (−1.2 to 1.8 kb) in the CAT reporter gene construct, we speculate that one or more PAX6 co-repressors may be involved in the regulation of *FABP7* expression. As PAX6 is a multifunctional regulator of both embryonic and adult neurogenesis, it controls expression of numerous downstream molecules involved in different developmental processes in the CNS [10,11]. Whether PAX6 regulates FABP7 expression through direct binding to its promoter region or through cooperation with other factors will be the subject of future studies.

PAX6 is downregulated in grade IV astrocytomas compared to grade III astrocytomas and normal brain tissue [19]. PAX6 has also been reported to be expressed at elevated levels in glioma cells with low tumorigenic potential compared to highly malignant tumors, and low levels of PAX6 correlate with a poor prognosis [18]. Furthermore, functional studies suggest that PAX6 suppresses growth, invasiveness and angiogenesis of glioblastoma cells *in vitro* and tumor growth *in vivo* [18,19,30,31]. Like PAX6, FABP7 suppresses malignant glioma cell growth *in vitro* [4]. However, FABP7 has also been shown to promote cell migration in malignant glioma [4,32] and expression of FABP7 in the nucleus has been associated with unfavorable patient outcome in grade IV astrocytoma [6,32]. Thus, the subcellular distribution of FABP7, rather than overall protein levels, may determine FABP7 function in malignant glioma [6,7]. Furthermore, PAX6 target genes in addition to *FABP7*, such as the matrix metalloproteinase-2 (*MMP2*) implicated in genesis and progression of malignant gliomas [30], likely affect the growth properties of malignant glioma cells.

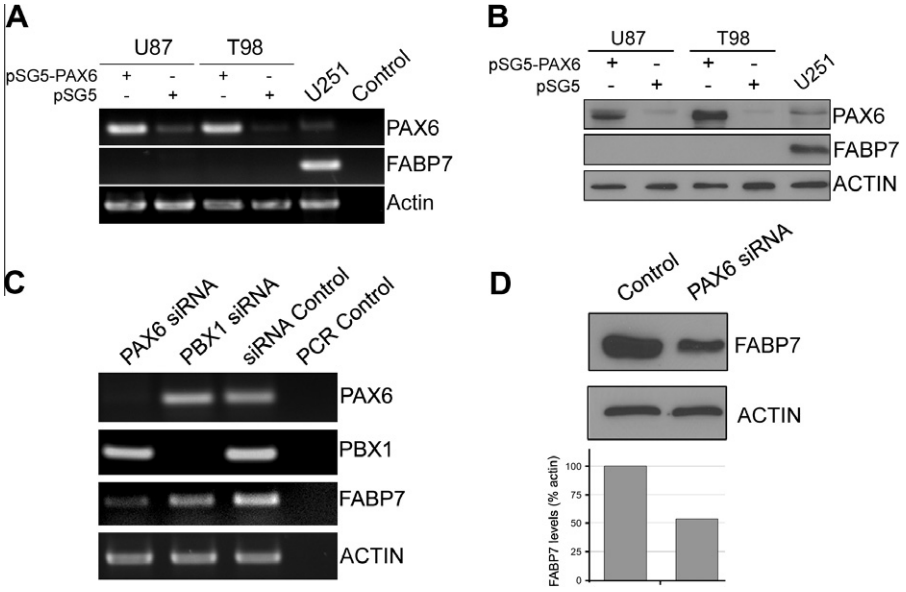

Fig. 4. Modulation of FABP7 expression by manipulation of PAX6 levels in human malignant glioma cells. (A) Total RNA (5 μg) from transfected U87 and T98 cells, as well as from FABP7-positive U251 cells, were reverse-transcribed and PCR-amplified with PAX6, FABP7 and actin primers. (B) Western Blot analysis of whole cell lysates from U87 and T98 cells transfected with the PAX6 expression construct or pSG5 empty vector. The filter was sequentially immunostained with anti-PAX6 (upper panel), anti-FABP7 (middle panel) or anti-actin (lower panel) antibodies. U251 cells served as the positive control for FABP7 immunostaining. (C) RT-PCR analysis of PAX6, PBX1, FABP7 and actin in M103 cells transfected with PAX6, PBX1 or control siRNAs. (D) Western Blot analysis of FABP7 and actin in whole cell lysates prepared from M103 cells transfected with PAX6 siRNA or control siRNA. Bands were quantitated using the Adobe Photoshop densitometry histogram function.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.05.019.

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