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Yes-associated protein promotes tumour development in luminal epithelial derived breast cancer

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ABSTRACT

Yes-associated protein (YAP) is inactivated by the tumour suppressing Hippo pathway. The YAP gene is amplified in human liver cancer, and promotes tumour growth. However, there are contrasting reports about its function in breast cancer. Studies have demonstrated both oncogenic or tumour suppressor functions. Our study aims to clarify the role of YAP in breast cancer. We investigated the expression of YAP in 69 cases of human breast cancer tissue by immunohistochemistry (IHC). The role of YAP on cell growth *in vitro* and tumourigenesis *in vivo* were evaluated. We found that YAP was expressed in 75.4% (52/69) of breast cancer samples; amongst these cases YAP was overexpressed in 29% (20/69). There was no YAP expression in the remainder (17/69) cases. Breast cancer cell lines in which YAP was either overexpressed or depleted confirmed that YAP markedly promotes cell proliferation. This was confirmed *in vivo:* overexpression of YAP enhanced tumour formation and growth, whereas downregulation of YAP decreased the tumour formation and growth. Our results suggest that YAP acts as an oncogene in a subtype of breast cancer.

1. Introduction

Yes-associated protein is a candidate oncogene regulated by the Hippo pathway, a novel tumour suppressor pathway first characterised in Drosophila. Components of the Hippo pathway are highly conserved in mammals. YAP phosphorylation mediated by the Lats tumour suppressor kinase (the mammalian homologue of Wts) inhibits its function.[1–3] This regulation of YAP by Lats is implicated in cell contact inhibition and tissue growth control.[3,4] The YAP gene is located on human chromosome 11q22, which is frequently amplified in several human cancers,[5,6] and is also a site of frequent loss of heterozygosity (LOH) in breast cancers.[7–11] YAP expression is elevated in multiple types of human cancer.[3,6,12,13] In nude mice, YAP acts synergistically with myc oncogene expression to enhance tumour growth.[6] In mice transgenic for the overexpression of the YAP gene, the liver increases in size and eventually leads to tumour development.[12,14] Overexpression of YAP in the breast cell line MCF10a induces epithelial–mesenchymal transition (EMT), which is associated with metastasis.[15] However, its role in breast cancer appears to be consistent with a tumour suppressor. In luminal breast cancer, YAP expression is lost in 63.6% and likewise in infiltrating ductal carcinomas, 63.4% of cases showed loss of YAP expression. Short hairpin RNA (shRNA) mediated knockdown of YAP in breast cancer cell lines suppressed anoikis, increased migration and invasiveness. YAP knock down also inhibited the response to taxol, and enhanced tumour growth in nude mice.[16]

We aimed to further delineate the role of YAP in breast cancer.

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2. Materials and methods

2.1. Cell culture

Human breast cancer cell lines MDA MB 453, MDA MB 361, MCF-7, ZR 75-1, SKBR-3, T47D, BT 483 and BT 474 were obtained from the American Type Culture Collection (ATCC) and maintained as recommended. The immortalised normal breast epithelium cell line HB4A was kindly provided by Dr. Subham Basu's group (Queen Mary University of London) and cultured as described previously.[17] Murine normal mammary cell line HC11 and mammary tumour cell lines JC and 410.4 were also kind gifts from Dr. Subham Basu's group and were cultured in DMEM with 10% FBS. Control and YAP shRNA stable cell lines were made by transfection with either control vector PRS or YAP shRNA expressing vector PRS-YAP, respectively (see below), and selected with 5 μg/ml puromycin (Invitrogen). The target sequence of YAP shRNA was described previously.[18] Stable cell lines overexpressing pcDNA3.1(+) vector or pcDNA3.1-YAP were generated by transfection of 410.4 cells and selected with 500 μg/ml G418 (Invitrogen).

2.2. Plasmids

The pcDNA3.1(+) vector was purchased from Invitrogen. cDNA was synthesised from RNA extracted from MCF-7. The YAP1 gene was amplified by PCR from cDNA using the following primers: the forward primer contains an EcoRI restriction site as underlined 5′-GAATTC GAGGCAGAAGCCATGGAT–3′; the reverse primer contains an XbaI restriction site as underlined 5′-TCTAGA GCTCTATAACCATGTAAGAAAGCT–3′. Following restriction digests, the YAP gene was cloned into the corresponding EcoRI and XbaI sites of the pcDNA3.1(+) vector. DNA sequencing confirmed the insertion. Plasmids were purified using Qiagen Plasmid Maxi kit.

2.3. Transfection

All the transfections were performed using the Effectene transfection kit (Qiagen) according to the manufacturer's instructions.

2.4. Immunohistochemistry (IHC)

Paraffin sections of normal and tumour tissue were stained for YAP expression using YAP antibody (H-125, Santa Cruz, 1:40), and counterstained with haematoxylin. All tissue was collected in accordance with local institutional board policy. All patients gave written informed consent as required by the local ethics committee of our hospital. The staining of YAP was scored independently by two pathologists, the scale of scores of YAP expression in cancer tissues was recorded as negative, no difference and weaker expression and stronger expression (overexpression) as compared to normal breast tissues.

2.5. Immunofluorescence (IF) staining

Cells were grown on coverslips in 6-well plates. Cells were fixed with 4% paraformaldehyde (Sigma) 4% (v/v) in PBS for 20 min at room temperature. Then cells were permeabilised in 0.1% Triton X-100 (Sigma) in PBS for 15 min at room temperature. IF staining of YAP was performed using YAP antibody (H-125, Santa Cruz, 1:40), and cell nuclear was counterstained with DAPI (300 ng/mL, Invitrogen). Staining was inspected on a Zeiss LSM 510 Meta confocal microscope, and pictures were captured.

2.6. Cell growth assay

Log-phase cells were plated in 2 ml of medium per well of a 6 well plate (in triplicate). From day 2 after plating cells, cells were trypsinised and counted using a Coulter counter for 4 days, and the total number of cells was recorded.

Experiments were performed three times independently.

2.7. Western blot analysis

Equal amounts of protein extracts were run in 10% SDS–PAGE and subsequently transferred to a PVDF membrane (Hybond-P, Amersham Pharmacia Co.). Anti-YAP antibody (H-125) (Santa Cruz, 1;200), mouse anti-human proliferating cell nuclear antigen (PCNA) antibody (Abcam, 1:1000), anti-actin antibody (Sigma, 1:1000), anti-c-JUN antibody (Cell Signalling, 1:1000), anti-cyclin E antibody (C-19) (Santa Cruz, 1;200), anti-cyclin D1 antibody (C-20) (Santa Cruz, 1;200) were used as indicated. Membranes were dried and then incubated for 1 h at room temperature with the antibodies. After washing the membranes were incubated for 1 h at room temperature with secondary horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies (Amersham Pharmacia) diluted at 1:1000 in TBS-0.01% Tween 20. Finally, the blots were detected with the ECL system (Amersham Pharmacia) and membranes were exposed to X-ray film.

2.8. Cell fractionation

Cells were collected by trypsinisation when cells reached 70–80% confluence. Cell fractionation was carried out using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific) according to the manufacturer's instructions. Equal amounts of proteins from nuclear fraction and cytoplasmic fraction were loaded on 10% SDS–PAGE and subsequently the expression of YAP was detected by western blot analysis.

2.9. Real-time quantitative PCR (q-PCR)

RNA was extracted from cells using TRIzol reagent (Gibco) according to the manufacturer's instructions. Reverse transcription was performed using oligo (dT)12–18 and SuperScript II RT (Invitrogen) according to the manufacturer's instructions. The real-time PCR were carried out using SYBR Green PCR master mix (Applied Biosystems) and the products detected with the ABI 7700 Sequence Detector (Applied Biosystems). YAP primers' sequences and GAPDH primers were described previously.[16]

2.10. Tumour growth in mice

Five female BALB/c SCID mice aged 4–6 weeks were used in each of the following groups: 1×10^7 MCF-7 PRS or PRS-YAP

expressing cells were injected subcutaneously in the flanks. Eight BALB/c mice aged 4–6 weeks were used in each of the following groups: 2×10^6 410.4 pcDNA3.1 or pcDNA3.1-YAP cells were injected subcutaneously in the flanks. Tumour was measured twice a week and tumour volume was calculated as Volume = (Length × Width^2 × 3.1415926)/6. Mice were sacrificed when tumour size reached 1.2cm × 1.2cm. Animal experiments were approved by the local ethics committee of our hospital.

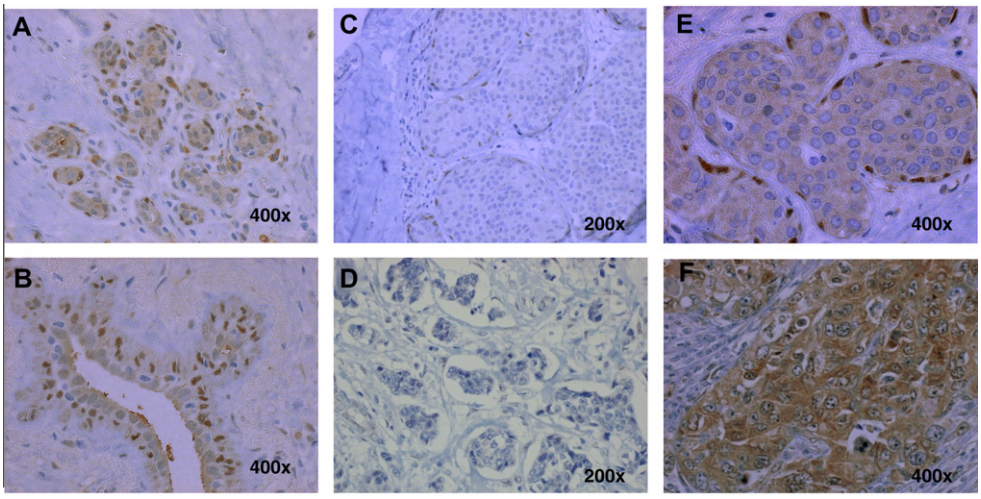
2.11. Statistical analysis

Student's *T* and chi-square tests were performed as indicated in the figures. Results were considered significant at $P \leqslant 0.05$.

3. Results

3.1. YAP expression in breast cancer

YAP expression is elevated in liver and other types of cancers. In contrast, its expression was reported to be lost in 60% of breast cancers, partly due to LOH (16). Because data on YAP gene expression in breast cancer is limited, we decided to examine the expression of YAP protein in further human breast cancers tissue. YAP gene expression was examined in tissues of normal breast ($n = 5$) and breast luminal epithelial derived cancer tissue ($n = 69$: 10 cases of lobular cancer and 59 cases of ductal cancer) by immunohistochemistry (IHC). In normal breast, strong nuclear presence of YAP was demonstrated in myoepithelial cells, with weaker cytoplasmic and



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Histology	sample number (n)	YAP expression: negative	variable	overexpression
LCIS	10	2	6	2
* DCIS	29	7	14	8
* IDC	30	8	12	10
+ I	10	3	5	2
+ II	10	2	3	5
+ III	10	3	4	3
		24.6% (17/69)	46.4 (32/69)	29% (20/69)

Fig. 1 – Yes-associated protein expression in normal breast and breast cancer. YAP expression was detected by IHC using YAP antibody (1:40). YAP expression in normal lobular (A) and normal duct (B). YAP is positive in the nucleus of all myoepithelial cells; While YAP is positive in cytoplasm of luminal cells. (C) Representative staining of DCIS showing YAP expression is absent in tumour cells. YAP expression is also lost in myoepithelial cells. (D) Representative example of IHC observed in IDC. YAP expression is lost in tumour cells. (E) Representative example of IHC observed in DCIS. YAP is positive in the cytoplasm of tumour cells as well as in surrounding myoepithelial cells. (F) Representative example of overexpression of YAP observed in IDC. YAP is strongly expressed in the cytoplasm of tumour cells. (G) Summary of IHC of YAP in stages of breast cancer. Chi-square test was performed. $^*P > 0.05$; $^+P > 0.05$.

nuclear levels in the luminal epithelial cells of lobular (Fig. 1A) and ductal tissue (Fig. 1B). In ductal carcinoma *in situ* (DCIS) and invasive ductal carcinoma (IDCS), there was variability in levels of YAP expression (Fig. 1C and D). 44% (26/59) of the cases demonstrated similar expression levels to normal breast tissues, 25.4% (15/59) cases were negative for YAP expression (Fig. 1E). However 30.5% (18/59) cases showed overexpression as compared to normal tissue (Fig. 1F). In lobular carcinoma, the expression of YAP revealed a similar pattern to that in ductal carcinoma. YAP expression was similar to normal lobular tissue in 60% (6/10), it was lost in 20% (2/10) of cancers and overexpressed in the remaining 20% (2/10) of cases. Overall, YAP expression was negative in 24.6% (17/69); were at a similar level to normal tissue in 46.4% (32/69) and overexpressed in 29% (20/69) (Fig. 1G). The status of YAP expression was not correlated to the grade of the breast cancers.

3.2. *YAP expression in breast cancer cell lines*

In order to characterise the role of YAP in the development of breast cancer, we needed breast cancer models. YAP expression was detected by western blot in a panel of human breast cancer cell lines along with a normal breast cancer cell line (HB4A) (Fig. 2A). YAP is weakly expressed in HB4A cells; while YAP is highly expressed in MDA MB453, MCF-7, SKBR3, T47D and BT474 cell lines. In MDA MB361 cells YAP is expressed at the similar level to HB4A cells. Next, we investigated if YAP overexpression is regulated at the level of transcription by examining the abundance of mRNA using real-time quantitative PCR (Fig. 2B). As shown in most cell lines the level of YAP mRNA correlated with the level of the protein, implying YAP expression is regulated at gene transcription. To investigate the subcellular localisation of YAP, western blot was performed

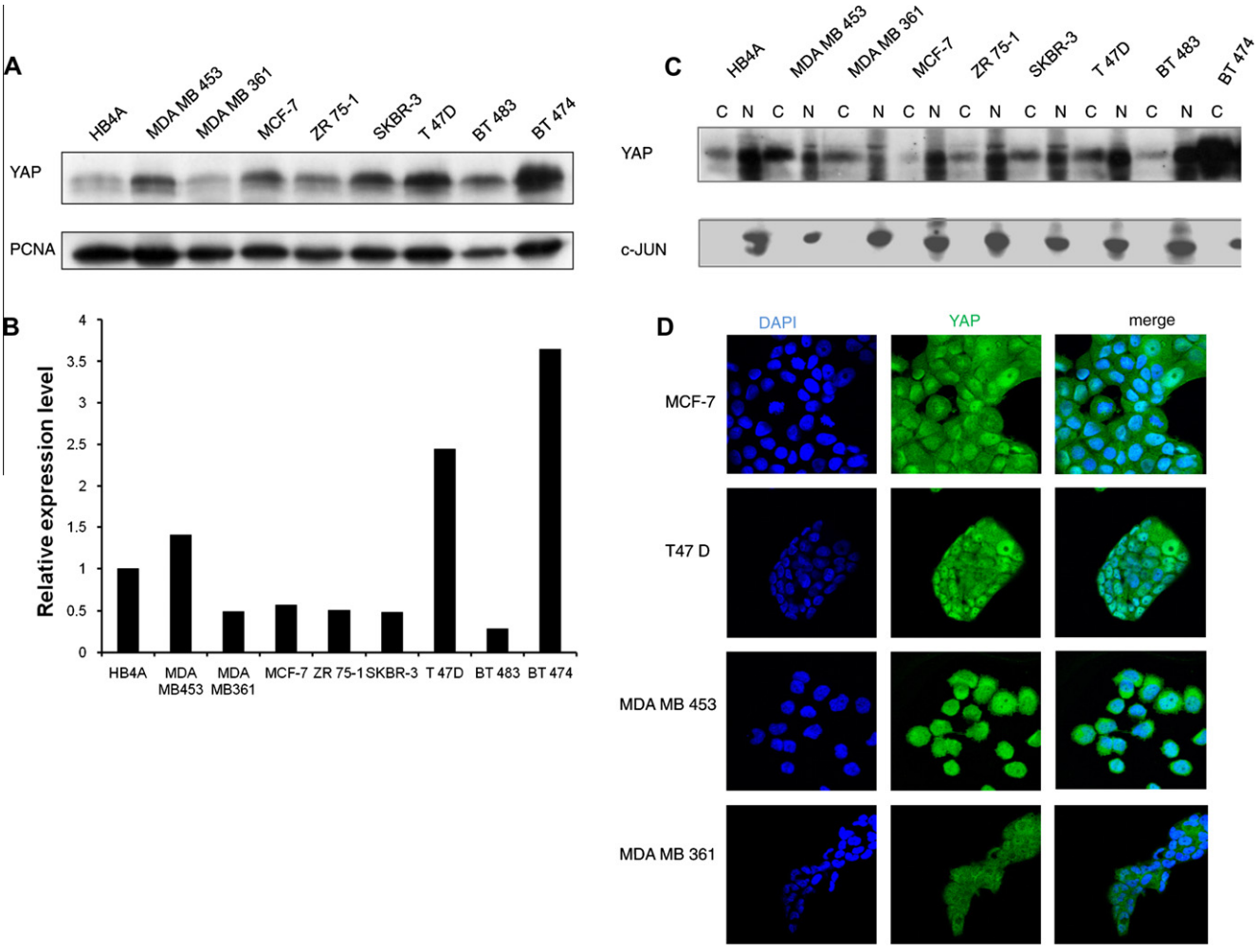

Fig. 2 – Yes-associated protein expression in breast cell lines. (A) YAP expression was detected in the normal breast cell line HB4A and breast cancer cell lines by western blot, PCNA was used as a loading control. (B) YAP expression was measured by real-time PCR in breast cell lines. Average level of expression is shown. (C) Cells were fractionated; YAP protein was detected by western blot in cell fractions (C = cytoplasm and N = nucleus). c-JUN was used as control for nucleus fractions. (D) Immunofluorescence (IF) staining of breast cancer cells lines. Cells were stained with YAP antibody (green) and cell nuclear was stained using DAPI (blue). Pictures were captured with 60× lens on the confocal microscope.

on fractionated cells. YAP is mainly localised in nucleus in most of the cell lines except in MDA MB453 and BT 474 cells, in which the majority of YAP was seen in cytoplasm, although there is still a significant amount of protein in the nucleus (Fig. 2C). This localisation was further confirmed by immunofluorescence staining of YAP in the representative cell lines (Fig. 2D). We also measured YAP expression in murine cell lines (Fig. 3B): YAP expression was not detected by western blot in non-transformated mammary epithelia cells (HC11) but was in JC and 410.4 tumour-derived mammary epithelia cell lines.

3.3. *YAP promotes cell growth*

To examine whether YAP expression affects breast cancer cell growth, we generated human breast cancer stable cell lines in MCF-7 cells, transfected with either a pRetroSuper-derived short hairpin RNA (shRNA) vector targeting YAP (PRS-YAP) or the control vector (PRS). YAP protein expression was suppressed in the stable cells as demonstrated by western blot in Fig. 3A by western blot. These stable cell lines were plated and cells were counted each day for four days to establish cell growth curves. Suppression of YAP by YAP shRNA impeded cell growth (Fig. 4A). Cell cycle progression was analysed in these cells. After the cell cycle released from G1 arrest following aphidicolin treatment, cell cycles progressed faster in MCF-7 PRS cells than in PRS-YAP cells as shown at 4 h time-point, showing fewer cells at G1 phase in PRS cells compared to PRS-YAP cells and showing fewer cells at G2/M phase in PRS cells compared to PRS-YAP cells at 12 h time-point (Supplement Fig. 1A and B). To evaluate whether overexpression of YAP is able to drive cell growth, we established murine 410.4 stable cell lines expressing pcDNA3.1 vector or pcDNA3.1-YAP. YAP overexpression was confirmed by western blot (Fig. 3C). Overexpression of YAP significantly increased cell growth in 410.4 cells (Fig. 4B) over a 4-day period. It has been reported that over-expression of YAP increases cell growth in other cell systems through upregulation of cyclin E.[6,19] In MCF-7 cells and 410.4 cells YAP however does not regulate cyclin E expression (data not shown), and cyclin E expression does not change in other breast cell lines upon the suppression of YAP expression (Supplement Fig. 2). However, cyclin D1 expression was down regulated in YAP suppression cells and up regulated in YAP overexpression cells (Fig. 4C, Supplement Fig. 2). Therefore, YAP may promote cell growth through up regulation of cyclin D1 expression.

3.4. *YAP enhances tumour growth in vivo*

To investigate the role of YAP expression in tumour growth *in vivo*, we used human and murine breast cancer cell lines with suppressed and overexpressed YAP expressions, MCF-7 and 410.4, respectively. MCF-7 PRS-YAP cells and MCF-7 PRS control cells were subcutaneously injected into nude mice and the tumour growth was observed. Tumours were formed only in mice injected with cells in which YAP was present, while no tumour was formed in mice injected with YAP suppressed cells (Fig. 5A and B). 410.4 pcDNA3.1-YAP cells and 410.4 pcDNA3.1 control cells were subcutaneously injected into BALB/c mice. Tumours formed earlier and grew faster in mice injected with cells in which YAP was overexpressed (Fig. 5C). Tumours were grown in all the mice injected with 410.4 pcDNA3.1-YAP while very few mice formed tumours injected with control cells (Fig. 5D). YAP therefore enhances tumour formation and growth *in vivo* in these models.

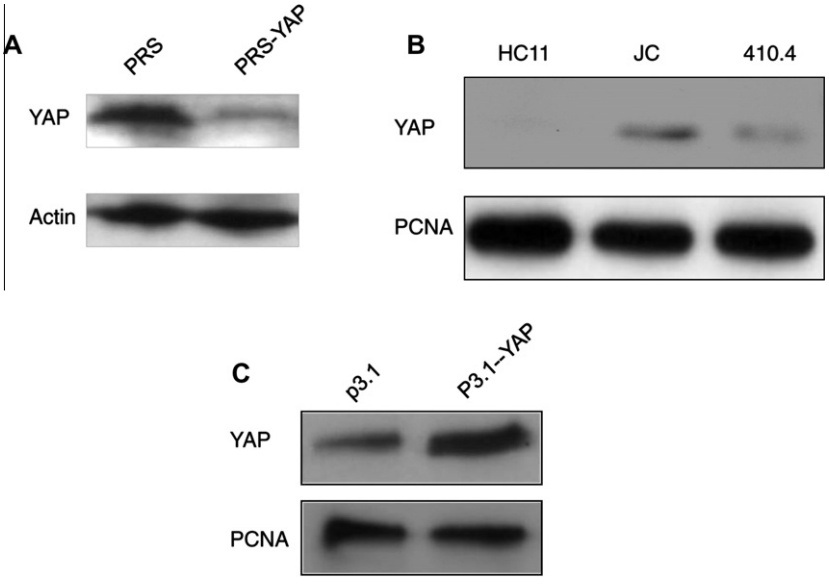

Fig. 3 – Stable cell lines were established. (A) YAP expression was determined by western blot in MCF-7 stable cell lines expressing vector control PRS or YAP shRNA PRS-YAP. PCNA was used as a loading control. (B) YAP expression was detected in murine normal mammary cell line HC11 and mammary tumour cell lines JC and 410.4 by western blot, PCNA was used as a loading control. (C) YAP expression was measured by western blot in stable 410.4 cell lines expressing control vector p3.1 (pCDNA3.1) or p3.1-YAP, PCNA was used as a loading control.

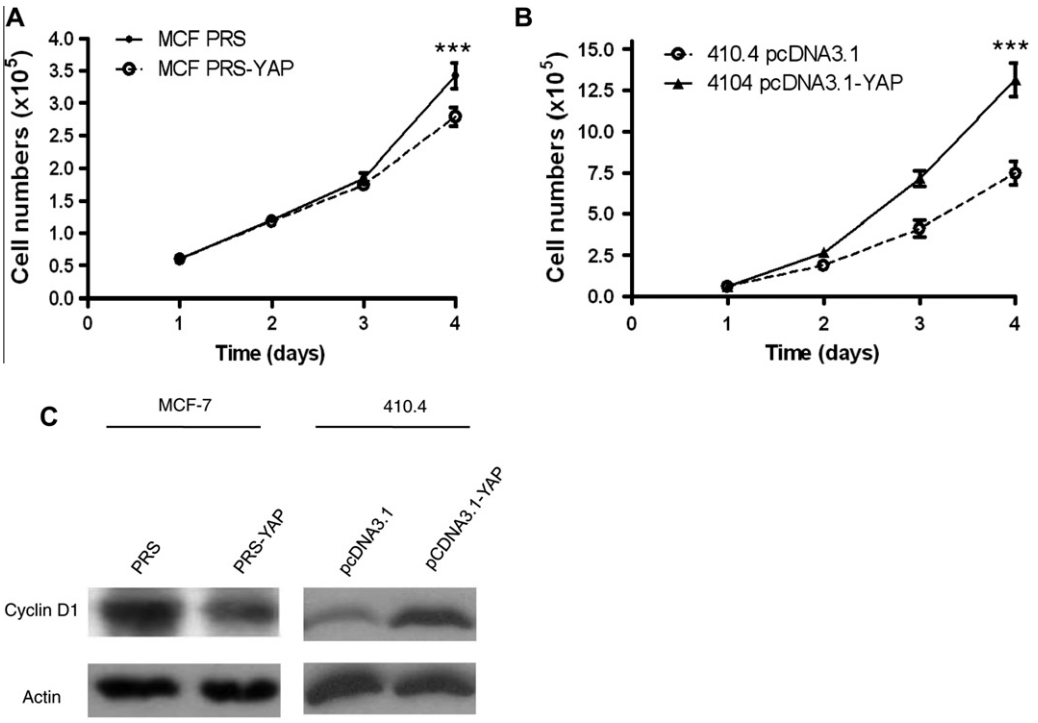

Fig. 4 – Yes-associated protein expression promotes cell growth. (A) Cell growth curve was established in MCF-7 PRS (solid line) and MCF-7 PRS-YAP (dashed line) cell lines. Cell numbers were counted at indicated time-points. (B) Cell growth curve was established in 410.4 p3.1 (dashed line) and 410.4 p3.1-YAP (solid line) cell lines. Cell numbers were counted at indicated time-points. Results represent one of three independent experiments; Error bars represent ±SD. Student's *T* test was performed. $^{***}P < 0.01$. (C) Cyclin D1 expression was analysed in stable cell lines. Actin was used as a loading control.

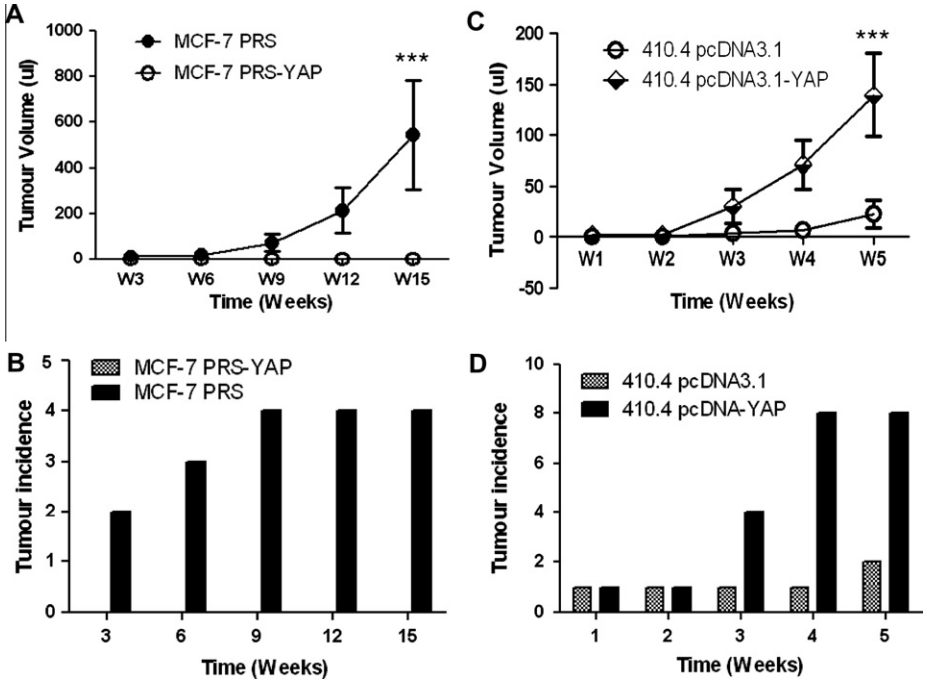

Fig. 5 – Yes-associated protein expression promotes tumour progression in mice. (A) Tumourigenicity of control and YAP shRNA transduced MCF-7 cells were injected subcutaneously into flank of BALB/c nude mice ($n = 5$), was assessed by tumour volume over indicated time. (B) Tumour incidence was recorded (C) Tumourigenicity of control and YAP expressed 410.4 cells were injected subcutaneously into flank of BALB/c mice ($n = 8$), was assessed by tumour volume over indicated time. (D) Tumour incidence was recorded. Error bars represent ±SD. Student's *T* test was performed. $^{***}P < 0.01$.

4. Discussion

We have demonstrated that upregulation of YAP increases growth in breast cancer cell lines *in vitro* and also promotes the growth of such tumours in syngenic and xenograft *in vivo* models. YAP is overexpressed in a number of luminal epithelial derived breast cancers.

Compared to normal breast tissue (ductal and lobular), we found YAP was overexpressed in 29% (20/69) of breast tumours and with loss of expression in 24.6% (17/69). The remainder demonstrated a similar pattern to normal breast tissue. Yuan et al. showed YAP expression was lost in more than 60% of the breast cancer tissue.[16] In that study, similar to ours the loss of YAP expression was not correlated to disease grade. No overexpression of YAP was identified in Yuan's study. Differences in the expression of YAP in breast cancers between the studies are likely to be due to heterozygosity of the clinical samples. YAP is overexpressed in human liver cancer, secondary to gene amplification.[6] However, YAP gene amplification was not found in breast cancer.[15] In this work, YAP overexpression in breast cancer cell lines appear to be regulated at the level of transcription as demonstrated by real-time PCR assay (Fig. 2).

Unlike in liver cancer cell lines, YAP does not regulate the expression of cyclin E in the breast lines (MCF-7 and 410.4 and other cell lines). However, we found that YAP up regulates cyclin D1 expression through unknown mechanisms; it could contribute to the enhanced cell growth in YAP expressed cells. Although YAP expression in MDA MB231 cells[16] suppressed growth, it is not a typical luminal epithelial derived breast cancer cell line. MDA MB231 shows mesenchymal cell characteristics. Overholtzer et al.[15] showed YAP had oncogenic function in a model based on the non-transformed mammary myoepithelial-derived cell line, MCF10A. Therefore, use of a MCF10a cell model may interpret the functions of YAP in this type of cancer. The MCF-7 breast cancer cell line is derived from human luminal epithelial tissue. Therefore this and other similar cell lines may more accurately reflect luminal epithelial derived breast cancers, the majority of human breast cancers.

YAP binds to transcription factors such as TAp73, regulating their function.[18] The function of YAP itself is controlled by upstream signalling pathways. Akt phosphorylates YAP at Serine127 enabling it to bind to 14-3-3. This complex is restricted to the cytoplasm and thus cannot regulate gene transcription. In Drosophila, the YAP homologue, yorkie, is crucial for organ size and proliferation. In this species it acts downstream in the HIPPO/WTS pathway.[19] YAP also functions in the HIPPO/WTS human homologue MST2/LATS.[20] In mammalian cells, YAP function is inhibited by enhanced cell density acting via this pathway. In this case, phosphorylation by the Lats tumour suppressor kinase leads to cytoplasmic translocation and inactivation of the YAP oncoprotein.[3] Furthermore, subcellular localisation of YAP, as in our study, varies in different cell types. Its function may be different in these compartments which may allow it to take on a tumour suppressor role or an oncogenic role, which needs to be addressed in future work.

Conflict of interest statement

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2011.10.001.

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