The tumour suppressor PTEN is a key negative regulator of the PI3K-Akt pathway, and is frequently either reduced or lost in human tumours. Murine genetic studies have confirmed that reduction of Pten promotes tumourigenesis in multiple organs, and demonstrated dependency of tumour development on the activation of downstream components such as Akt. Insulin-like growth factors (IGFs) act via IGF1R to activate the PI3K-Akt pathway, and are commonly upregulated in cancer. A context-dependent interplay between IGFs and PTEN exists in normal tissue and tumours; increased IGF2 ligand supply induces Pten expression creating an autoregulatory negative feedback loop, whereas complete loss of PTEN may either cooperate with IGF overexpression in tumour promotion, or result in desensitisation to IGF ligand. However, it remains unknown whether neoplasia associated with Pten loss is dependent on upstream IGF ligand supply, as lack of Igf2 results in extended survival and delayed tumour development while biallelic supply is associated with reduced lifespan and accelerated neoplasia in females. Furthermore, we demonstrate that reduction of PTEN protein to heterozygote levels in human MCF7 cells is associated with increased proliferation in response to IGf2, and does not result in desensitisation to IGF2 signalling. These data indicate that the effects of Pten loss at heterozygote levels commonly observed in human tumours are modified by Igf2 ligand, and emphasise the importance of the evaluation of upstream pathways in tumours with Pten loss.

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Keywords: PTEN; IGF2; Cowden syndrome; imprinting; placenta; mammary gland

Introduction

PTEN is a dual specificity phosphatase that acts as a key negative regulator of the ligand-activated PI3K-Akt pathway (Carracedo and Pandolfi, 2008). PTEN acts to dephosphorylate phosphatidylinositol (3,4,5) triphosphate to the diphosphate (4,5), thus reducing activation; placenta; mammary gland

Insulin-like growth factor 2 (IGF2) is a potent embryonic growth factor with homology to IGF1 that signals through IGF1R and insulin receptor isoform A to activate the PI3K-Akt and mitogen-activated protein kinase pathways (Foulstone et al., 2005). The supply of IGF2 ligand is tightly regulated, with one mechanism being genomic imprinting of Igf2 both in human and
mouse. Imprinting restricts *Igf2* expression to the paternal allele with reciprocal maternal expression of a non-coding RNA (ncRNA) *H19* (Bartolomei et al., 1991; DeChiara et al., 1991). Coordinate expression of *Igf2* and *H19* on each allele is regulated by a differentially methylated intergenic imprinting control region (ICR) and competition for shared downstream enhancers between the two loci (Bell and Felsenfeld, 2000). Loss and gain of function of *IGF2* contribute to the human growth disorders Silver-Russell (OMIM #180860) and Beckwith-Wiedemann (OMIM #130650) syndromes, respectively. In the mouse, paternal inheritance of an *Igf2* null allele (*Igf2*−/−) is associated with approximately 1% wild-type (wt) levels of *Igf2* expression and results in foeto-placental growth restriction (60% wt) (DeChiara et al., 1991). In contrast, maternal inheritance of a 13 kb *H19*/IGF2 deletion (*H19*−/−) results in both loss of ncRNA and biallelic *Igf2* expression. *Igf2* mRNA levels of 127–219% wt and foetal (127%) and placental (140%) overgrowth (Leighton et al., 1995). Loss of imprinting of *IGF2* is common in human cancers (van Roozendael et al., 1998; Cui et al., 2002) and murine studies have confirmed that *Igf2* is required for tumour progression in the RIP-TAg pancreatic (Chris-tofori et al., 1994), *ApcMm* intestinal (Hassan and Howell, 2000) and the *Pich/+* medulloblastoma models (Corcoran et al., 2008), whereas biallelic *Igf2* supply in combination with *ApcMm* promotes adenoma progression (Sakatani et al., 2005; Harper et al., 2006). Recently, growth regulatory and tumour suppressive roles for *H19* have been established (Yoshimizu et al., 2008; Gabory et al., 2009), and tumour-promoting miRNA (micro RNA) within the *Igf2* (miR-483*) and *H19* (miR-675-3p) loci reported (Tsang et al., 2010; Veronese et al., 2010).

As a negative regulator of the PI3K-Akt pathway, PTEN performs a pivotal role in the regulation of *IGF2* signalling, and several lines of evidence indicate a complex interplay between *IGF2* and PTEN in normal tissues and tumours. *Pten* expression is induced in response to *IGF2* ligand supply, generating negative feedback loops dependent on Egr1 and IGFBP2 (Moorehead et al., 2003; Perks et al., 2007). Moreover, evidence in human glioblastomas with PTEN loss indicates that *IGF2* overexpression promotes tumour growth, suggesting that the two may cooperate in tumour development (Soroc-weana et al., 2007). However, desensitisation of *IGF2* signalling can occur following either PI3K activation or PTEN loss through feedback mechanisms that include regulation of *IGF1R* levels, insulin receptor substrate (IRS)-1 stability, regulation of IRS2 and inhibition of IRS-1 by protein kinase C (PKCz) (Liu et al., 2001; Ravichandran et al., 2001; Lackey et al., 2007). Collectively, these data suggest that although subtle changes in *Igf2* and *Ptten* expression may autoregulate through feedback mechanisms, such compensation is inadequate to prevent the effects of allelic dosage variation of either gene in the mouse (DeChiara et al., 1991; Leighton et al., 1995; Di Cristofano et al., 1998; Podsypanina et al., 1999) and raise the possibility that combined dysregulation may cooperate in tumour promotion. However, at present the in vivo dependency of *Pten*+/−-associated tumorigenesis on *Igf2* supply remains unclear. Here, we sought to address this question by the generation of *Pten*+/− mice with differing *Igf2* allelic dosage, resulting in compound mutants with either normal, substantially decreased or approximately doubled *Igf2* expression.

**Results**

*Igf2* and *Pten* regulate placental growth and organisation

We first evaluated the interactions of *Igf2* and *Pten* by mating mutant mice on a C57BL/6 background to generate wt, *Pten*−/−, *H19*−/−, and *H19*−/−; *Pten*+/− compound mutant progeny. The expected foeto-placental overgrowth of *H19*−/− mice was evident by E12.5 and persisted throughout gestation (Figures 1a and b). Unexpectedly, we also detected an increase in *Pten*−/− foetal and placental weights by E15.5 (119% and 123% wt, respectively, *P<0.001* both comparisons) that subsequently diminished in the foetus by E18.5 (107% wt, *P=NS*) but persisted in the placenta (122% wt, *P<0.001*). *H19*−/−; *Pten*+/− compound mutants were, in turn, larger than either class of single mutant (Figures 1a and b), though the combination was less than additive in the foetus (131% wt at E15.5 and E18.5) and greater than additive in the placenta (165% and 180% wt, *P<0.001* vs all groups) (Figures 1a and b).

An approximate doubling of *Igf2* mRNA in *H19*−/− and *H19*−/−, *Pten*−/−, *Pten*+/− mutant placentas was associated with a relatively smaller increase in protein (Figure 1c, Supplementary Figures S1a and b), whereas *Pten* expression and protein in *Pten* heterozygotes were half and two thirds wt level, respectively, and independent of *Igf2* (Figure 1d, Supplementary Figures S1c and d). As expected, the decrease in *Pten* protein was accompanied by an increase in Ser473-phosphorylated Akt (p-AktS473) in both *Pten*−/− and *H19*−/−; *Pten*+/− mutants (Figure 1d), though importantly, no additional effect of biallelic *Igf2* expression was detected on Akt and Erk 1/2 activation. We next evaluated localisation of the placental growth effects. The murine placenta can be divided into three zones; the part-maternally derived decidua; the junctional zone, comprising spongiosphloblast (Sp) and glycogen cells (Gly); and the labyrinth (Lab), which contains foetal and maternal vessels and is the site of nutrient exchange. Overgrowth of *H19*−/− placentas was largely associated with preservation of placental architecture, whereas *Pten*+/− mutants displayed tortuosity of the Sp/Lab interface by E15.5 with expansion of the junctional zone (31.48 ± 1.68% in wt) (Figures 1e and f).

Biallelic *Igf2* supply potentiated both *Pten*+/− placental phenotypes, with marked abnormality of the Sp/Lab border and greater increase in the size of the junctional zone (34.83 ± 3.49%, *P<0.05* vs wt) in *H19*−/−; *Pten*+/− placentas (Figures 1e and f). Gly cells are known to be *Igf2* dependent (Lopez et al., 1996; Carter et al., 2006; Esquilliano et al., 2009) and, as anticipated, were present in increased numbers at E18.5 in *H19*−/− placentas.
though interestingly at lower levels than in Pten−/− mutants (Figure 1f). This indication of Gly cell regulation by Pten was supported by an increased expression of the Gly cell marker Pcdh12 (Coan et al., 2006) in Pten+/− placentas (137% wt, P > NS) (Figure 1g). Combined dysregulation in H19−/−, Pten−/− placetas was accompanied by further increase in Gly cell number and Pcdh12 expression (154% wt, P < 0.05) (Figures 1f and g), and markedly abnormal distribution of Gly cells throughout the Lab and Sp was confirmed with staining by Periodic acid-Schiff (Figure 1e). Immunohistochemistry demonstrated an apparent reciprocal distribution for Pten and Igf2 in subcellular regions of the placenta with increased labelling for

Figure 1  Igf2 and Pten regulate foetoplacental growth, Sp and Gly cells. (a, b) Fetal (a) and placental (b) weights by embryonic stage on a C57BL/6 background (main panel) and on an F1 hybrid C57BL/6:129S2 background at E15.5 (insets). Numbers below columns indicate weights as % of wt, numbers on y axis in insets indicate weights in mg. (c) Igf2 immunoblot of littermate E15.5 placentas. Multiple bands correspond to the 22 kDa, 56 residue pro-peptide and the 11–17 kDa, 87 and 104 residue ‘big’ forms described in human, which undergo variable glycosylation. The mature 7.5 kDa 67-residue peptide was not detected in placental tissue. Numbers below bands indicate % of wt level (quantified by densitometry in Supplementary figure S1b), to the right represent molecular weights in kDa. (d) Immunoblot of littermate E15.5 placentas for indicated proteins. Numbers below bands indicate levels as % of wt (quantified by densitometry in Supplementary figure S1d). (e) Littermate placentas at E18.5. To the left are low magnification haematoxylin and eosin-stained sections, higher magnification panels to the right show staining with periodic acid Schiff. Sp and Lab are indicated, and the Sp/Lab interface highlighted (yellow line). Note disorganisation of Sp/Lab interface in Pten+/−/C0 and H19−/−/C0 mutants. periodic acid Schiff staining demonstrates persistent Gly cells in all mutant classes most prominent and abnormally localised in H19−/−/Pten−/− placenta (pink cells indicated by arrows, right panels). Images are representative of ≥5 placentas examined in each class. (f) Quantification of proportion of Sp and Gly as percentage of placenta determined by point counting. (g) Expression of Gly cell marker Pcdh12 in E15.5 placenta assayed by RT–qPCR. Error bars in (a, b, f, g) indicate ± s.e.m. from ≥5 biological replicates. *, ** and *** indicate P < 0.05, < 0.01 and < 0.001, respectively.
activated Akt in the Sp (Supplementary Figures S1e and f). Examination of proliferation in the Lab using ki67 was increased by E18.5 in placentas with biallelic Igf2 supply, consistent with their increased relative overgrowth in late gestation (Supplementary Figure S1g).

We next generated mice with varying allelic dosage of Igf2: null (Igf2−/−), monoallelic or biallelic expression and either wt or heterozygous Pten supply on a uniform F1 hybrid C57BL/6/129S2 background. This both produced the growth effects and confirmed that the Pten+/+ foetoaplental phenotype persisted despite near complete absence of Igf2 (Supplementary Figures S2a–c).

**Biallelic Igf2 supply combined with Pten+/− results in cardiac hyperplasia and neonatal lethality**

By postnatal day 10 on a C57BL/6 background, a deficit of H19−/−,Pten+/− compound mutants was evident (49% of expected, P=0.0001). This was greater in females than males (29% of expected, P<0.001 vs 64%, P = NS) (Supplementary Figure S3a). A significant and disproportionate increase in heart weights in H19−/−,Pten+/− female neonates (Supplementary Figure S3b) was absent at postnatal day 5, by which time the deficit was evident. Magnetic resonance imaging at E15.5 (Supplementary Figure S3c) demonstrated that the increased weight of H19−/−,Pten+/− hearts was mirrored by changes in biventricular volume and interventricular septal thickness (Supplementary Figures S3d and e). Additionally, two H19−/−,Pten+/− foetuses demonstrated a marked dilatation and thinning of the ventricular myocardium (Supplementary Figure S3c). Collectively, these data suggested an association between the cardiac abnormalities and lethality. Quantification of cardiomyocyte density demonstrated growth because of hyperplasia (Supplementary Figure S3f), and analysis confirmed absence of Igf2 (Supplementary Figures S2a–c).

**Igf2 modifies Pten+/− survival**

We next examined the effects of Igf2 on Pten+/− tumorigenesis. Large (>10 mm) external tumours developed significantly earlier in H19−/−,Pten+/− (median 232 days) than Pten+/+, or Igf2−/−,Pten+/− mice (median not reached, Figures 2d–f). The difference in tumour onset by Igf2 in Pten+/− males (Figure 2e) was significant only for comparison of Igf2 null with biallelic mutants. However, variation in females was larger and highly significant for all comparisons (Figure 2f). External tumours were predominantly hyperplastic lymph nodes, mammary carcinomas and, less frequently, lymphomas. Clinically detectable (>5 mm) Pten+/− lymphadenopathy was accelerated with biallelic Igf2 expression and delayed in the Igf2−/−,Pten+/− cohorts (Supplementary Figures S4a–c). Furthermore, histopathological analysis demonstrated a tendency to higher-grade lymphoid lesions with Igf2 gain of function (Supplementary Figures S4d–g).

We next focused on the effects of variation of Igf2 allelic supply on epithelial gene expression and tumorigenesis. RT–qPCR (reverse transcriptase-quantitative PCR) on mammary glands, endometria and prostates of virgin animals of 6–8-weeks age confirmed that although Igf2 expression was reduced to approximately 1% of the levels of E9.5 embryos, it remained detectable and was substantially reduced in Igf2−/− mutants (1–8% wt), and doubled in H19−/− mutants (178.9–200% wt) (Supplementary Figures S5a–c). H19 expression was greatly reduced in H19−/− mutants, though no significant change was found in tissues from Igf2−/− animals and no alteration in Pten expression with change in Igf2 supply was detected (Supplementary Figures S5d–f).

Clinically detectable mammary carcinomas in females occurred significantly later in Igf2−/−,Pten+/− mutants than both Pten+/+ and H19−/−,Pten+/− groups. These data were combined with systematic analysis of macroscopically normal female mammary glands (see Supplementary methods) to determine the true incidence and timing of mammary neoplasia. A trend towards fewer carcinomas in Igf2−/−,Pten+/− mutants (Figure 3a) was accompanied by a significant delay in tumour onset (Figure 3b). Carcinomas were histologically similar in all cohorts with prominent stromal component (Figure 3c), with no differences in proliferation by ki67 labelling (not shown). No metastases were detected in any group. No pathological changes were observed in Pten wt female (n = 6) or Pten+/+ male (n = 30)
mammary glands, irrespective of Igf2 dose. Endometrial hyperplasia was common in all in Pten+/− groups, though strikingly no case of progression to carcinoma was detected in Igf2+/−,Pten+/− females, in contrast to 5/24 (21%) Pten+/− and 4/23 (17%) H19−/−Pten+/− animals (Figure 3d). Thus, lack of Igf2 was associated with a significant delay in carcinoma onset (Figures 3e,f), although these animals were killed for other indications. Although murine prostatic intraepithelial neoplasia (mPIN) was near-universal in all Pten+/− classes, mPIN was detected significantly earlier in H19−/−Pten+/− males and there was a trend towards fewer Grade 4 lesions in the Igf2−/−Pten+/− cohort (Figures 3g–i). Ki67 index in neoplastic endometrial and prostatic lesions did not vary between groups, and patterns of Pten loss and Akt activation were similar (not shown). Though common in all Pten+/− mutants, intestinal polyps were significantly smaller in animals lacking Igf2 (Igf2−/−,Pten+/− 3.1 ± 0.3 mm; Pten+/− 4.0 ± 0.2 mm; H19−/−,Pten+/− 3.9 ± 0.2 mm, P < 0.05 vs Pten+/−). Phaeochromocytoma was fully penetrant in all Pten+/− groups.

Promotion of mammary tumorigenesis by Igf2 combined with decreased Pten protein

We next sought to examine the effect of Igf2 effect on mammary tumorigenesis. Despite the extended life span of Pten+/− females lacking Igf2, mammary tumours in this group remained smaller than other mutants (Figure 4a). Conversely, tumour multiplicity tended to increase with gain of Igf2 alleles (Figure 4b). Mammary hyperplasia, a preneoplastic lesion (Supplementary Figure S6a), was detected at similar frequency in females of all genotypes (Figure 3a), suggesting that Igf2 may promote the transition to carcinoma in Pten+/− females. RT–qPCR confirmed that Igf2 expression in mammary carcinomas corresponded with Igf2 allelic dose (Figure 4c). As in the normal mammary gland, H19 expression was substantially decreased in H19−/−,Pten+/− mutant carcinomas (Supplementary Figure S6b). Levels of Pten mRNA in tumours relative to wt mammary gland were reduced by a similar degree in all genotypes (P = NS) (Figure 4d). There was no evidence of feedback downregulation of Igf1r secondary to this (Supplementary Figure S6c), though interestingly Igf1

Figure 2 Igf2 allelic dose modifies survival and tumour development in Pten+/− mice. (a–c) Survival of Pten+/− mice by Igf2 allelic dosage. Percentage of total (a), male (b) and female (c) populations alive by time. No deaths occurred in Pten wt controls (n = 73). Note the female-predominant effect (c). (d–f) Incidence of large (>10 mm) external tumours. Percentage of total (a), male (b) and female (e) populations with tumours by time. The total includes only external tumours during the study period of 450 days and visceral tumours detected at necropsy at study endpoint. Tumours were predominantly hyperplastic lymph nodes, lymphomas and mammary carcinomas.
expression was low in Pten<sup>+/−</sup> and H19<sup>−/−</sup> mammary carcinomas (Supplementary Figure S6d). Notably, despite similar Pten expression, immunoblot analysis demonstrated a significant progressive decrease in tumour Pten protein with increasing Igf2 allelic dose (Figures 4e and f). Associated Akt phosphorylation was universal (Figure 4e, Supplementary Figures S6e and f), and a trend to greater Akt activation with Igf2 gain of function was mirrored by a greater nuclear exclusion of FoxO1, a readout of PI3K-Akt pathway activity (Supplementary Figure 6g). Immunohistochemistry demonstrated foci of epithelial Pten loss in a minority of advanced lesions only (Figure 4g), insufficient in extent to fully explain the relative reduction in total protein.

**Perturbation of miR-483<sup>*</sup> and miR-675-3p in Igf2<sup>−/−</sup> and H19<sup>−/−</sup> mutants**

Given the recent demonstration of tumour-promoting functions for miR-483 and miR-675, we next assessed whether their perturbation in Igf2<sup>−/−</sup> and H19<sup>−/−</sup> mutants was likely to have contributed to the modification of phenotype. Using E9.5 embryos, selected for their high expression of Igf2 and H19 (Burns and Hassan, 2001), we confirmed the expected changes in Igf2 expression in Igf2<sup>−/−</sup> and H19<sup>−/−</sup> embryos (0.8%, and 246.6% wt, respectively) (Figures 5a and b). We next examined changes in miRNA expression in both classes of mutant using Illumina 96-assay universal array matrix miRNA arrays.

miR-483<sup>*</sup> is located within the second intron of Igf2 in a region of the gene spared by the targeting construct used in this study (DeChiara et al., 1990). We anticipated an increase in miR-483<sup>*</sup> levels in H19<sup>−/−</sup> mutants of similar magnitude to that of Igf2, but surprisingly this was not the case (105.1% wt, P = NS). Also unexpected was an upregulation of miR-483<sup>*</sup> in Igf2<sup>−/−</sup> mutants (107.3% wt) (Figure 5c). Importantly, no correlation between Igf2 and miR-483<sup>*</sup> expression was detected ($R^2 = 0.365, P = NS$). miR-675-3p is located within the first exon of H19, which is deleted along with

![Figure 3 Igf2 allelic dose modifies Pten<sup>+/−</sup> epithelial neoplasia.](image-url)
10 kb of 5’ flanking sequence containing the DMR in the ΔH19/DMR mutant allele (Leighton et al., 1995). As expected, H19<sup>−/−</sup> mutants showed a significant decrease in miR-675-3p expression (78.1% wt, P < 0.001), though this was smaller than the reduction in H19 ncRNA (Figure 5d). Notably, miR-675-3p expression was unchanged in Igf2<sup>−/−</sup> mutants (99.6% wt, P = NS) (Figure 5d). Changes in the expression of 377 additional miRNAs analysed were modest and are listed in Supplementary Table S1. Aside from miRNA perturbations that we could attribute to expression of the neomycin resistance gene, we observed a number of

Figure 4  Igf2 promotes Pten<sup>−/−</sup> mammary tumourigenesis. (a, b) Mean mammary tumour volume (a) and mean number of tumour foci per animal (b) by Igf2 allelic dose. (c, d) Waterfall plots showing expression of Igf2 (c) and Pten (d) in mammary carcinomas (only two tumours in Igf2<sup>−/−</sup>Pten<sup>−/−</sup> mutants were of sufficient size to obtain tissue for RNA and protein analysis in addition to fixation). Horizontal lines indicate wt level (light grey) and mean expression of each mutant class (black), with percentage values relative to wt mammary gland. Expression was elevated >2-fold in 4/11 Pten<sup>−/−</sup> and 6/10 H19<sup>−/−</sup>Pten<sup>−/−</sup> mutants. (e, f) Immunoblot of mammary tumor lysates (e) showing progressive decrease in Pten protein with increasing Igf2 allelic dose, quantified by densitometry in (f). Numbers below bands in (e) indicate protein level as % of wt mammary gland from densitometry in (f) and Supplementary Figures S6e and f. Note the increase in p-Akt<sup>T308</sup> relative to p-Akt<sup>S473</sup>. (g) Immunohistochemistry demonstrated infrequent foci of complete epithelial Pten loss in advanced lesions only. Error bars in (a, b and f) represent ± s.e.m. Statistical comparisons in (c, d and f) are between Pten<sup>−/−</sup> mutant classes.
miRNAs deregulated to similar extents in mutants as miR-483* and miR-675-3p. Importantly, no miRNA significantly downregulated in Igf2⁻⁻ embryos demonstrated significant upregulation in H19⁻⁻ embryos, or vice versa.

Reduction of PTEN protein to heterozygote levels in human breast cancer cells associated with increased IGF2-mediated proliferation

PTEN loss has previously been shown to cause desensitisation to IGF1 and insulin signalling (Lackey et al., 2007). To reconcile these results with our data, and to examine the interaction between IGF2 and PTEN loss in a system free from H19 ncRNA, miR-483* and miR-675-3p perturbation, we used shRNA (small hairpin RNA) to reduce PTEN expression to heterozygote levels in MCF7 human breast cancer cells. PTEN protein in MCF7 cells with partial PTEN knockdown (MCF7PTEN KD) was 61% that of MCF7 cells expressing control shRNA (MCF7CTRL) (Figures 6a and b). As anticipated, reduced PTEN in MCF7PTEN KD cells was accompanied by increased Akt activation (Figure 6a). Importantly, no changes in levels of total IGF1R, total IRS1 (Figure 6a) or inhibitory Ser-612 phosphorylated IRS1 (not shown) were detected. Following IGF2 stimulation, both MCF7CTRL and MCF7PTEN KD cells demonstrated similar IGF1R-PI3K-Akt and mitogen-activated protein kinase pathway activation as demonstrated by comparable levels of

![Figure 6](image)

**Figure 6** Reduction of PTEN protein to heterozygote levels sensitises MCF7 cells to mitogenic effect of IGF2. (a) MCF7 human breast cancer cells with stable expression of either scrambled control (Ctrl) or anti-PTEN shRNAs were serum starved overnight and then stimulated with recombinant human IGF2 at 100 ng/ml. Lysates were prepared at baseline and after 2 hours following treatment, and immunoblotting for the proteins indicated performed. (b) Densitometry quantification of the reduction in PTEN protein (without IGF2 stimulation) from (a). (c) Proliferation of MCF7 cells expressing either control or anti-PTEN shRNA in serum-free medium following stimulation by IGF2. Exogenous ligand was indicated at the concentrations indicated, and absorbance measured after 48 h by MTS assay. Results are normalised to the values in controls treated with vehicle alone. Error bars indicate ± s.e.m. of at minimum six replicates per dose. Data in (a) and (b) are representative of experiments performed in duplicate and triplicate, respectively.
activated IGF1R (p-IGF1RY1131), IRS1 (p-IRS-1Y632), Akt (p-AktS473) and Erk1/2 (p-ErkT202/204) (Figure 6a).

It has also been shown in MCF7 cells that high concentrations of IGF2 result in relative inhibition of cellular proliferation through feedback induction of PTEN (Perks et al., 2007). We next assessed whether partial PTEN knockdown abrogated this autoregulation. Stimulation of MCF7CTRL and MCF7PTEN KD cells with increasing concentrations of IGF2 confirmed the mitogenic effect of recombinant ligand at concentrations of >1 ng/ml, and as anticipated in MCF7CTRL cells this plateaued at 100 ng/ml (Figure 6c). Notably, MCF7PTEN KD cells demonstrated increased proliferation in response to IGF2, most strikingly at high ligand concentrations, with no evidence of a plateau in proliferation (Figure 6c).

Human stromal interaction of IGF2 and PTEN
Finally, in light of the apparent promotion of stroma-predominant Pten+/− tumourigesis in order to evaluate the extent to which the effects of Pten loss in vivo are dependent upon upstream signalling. We report several novel findings with respect to development and tumourigenesis.

The Pten+/− placental phenotype we describe demonstrates tissue-specific requirements for biallelic Pten expression in mammalian development. Although both Pten+/− placental overgrowth and Sp disorganisation were increased by biallelic IGF2 supply, they were not ameliorated in IGF2 null mutants, and the discrete localisation of IGF2, Pten and p-Akt within the placental Lab are consistent with a complex interaction (Coan et al., 2006). However, when combined with the Akt1 null placental phenotype of small size, Sp and Gly cell deficiency (Yang et al., 2003), our data indicate that an IGF2-Pten-Akt axis regulates placental development (Lopez et al., 1996; Carter et al., 2006; Esquiliano et al.,...
As noted previously, the H19 ncRNA has recently been demonstrated to have tumour suppressive activity in vivo (Yoshimizu et al., 2008), whereas miRNAs in the IGF2 and H19 loci; miR-483 and miR-675 respectively, have both been ascribed tumour-promoting effects (Tsang et al., 2010; Veronese et al., 2010). Although Igf2 expression across the allelic series increased from 1 to 247%, there was no similar variation of any miRNA. Importantly, loss of H19 (<1% of wt) was not mirrored by equivalent reduction in miR-675-3p in H19 null mutants, and miR-483* expression appeared relatively unchanged. Disruption of miR-483* and miR-675-3p was minimal in Igf2 null animals, with no consistent pattern of H19 dysregulation detected. In addition to these changes, our global analysis shows that many independent miRNA are deregulated in both Igf2 null and H19 null mutants. Our data cannot exclude selective dysregulation of miR-483* and miR-675-3p in tumours, as the small number of Igf2 null, Pten null tumours available precluded meaningful analysis. Definitive in vivo assessment of the contribution of the miR-483* and miR-675-3p to phenotype may only be provided by specific conditional deletion of respective loci that modify miRNA expression independent of Igf2. However, our in vitro data demonstrate the proliferative effects of IGF2 at heterozygote levels of PTEN protein in a system free from H19, miR-483* and miR-675-3p perturbation.

IGF2 overexpression by human breast cancer stroma has been postulated to promote neoplastic growth by a paracrine mechanism (Singer et al., 1995). We demonstrate that increased Igf2 mRNA in stroma-prominent Pten null murine mammary tumours is mirrored by near-universal upregulation of IGF2 and downregulation of PTEN in human breast cancer stroma. The role of stromal Pten in suppression of mammary tumourigenesis has recently been demonstrated (Trimboli et al., 2009). Our data suggest that the role of stromal IGF2 in breast cancers merits further examination.

In summary, we show that variation of Igf2 ligand supply is associated with modification of development, survival and tumourigenesis in Pten null mice, that these changes are unlikely to result from perturbation of the H19 ncRNA, miR-483* or miR-675-3p, and that IGF2 ligand cooperates with partial PTEN loss to promote breast cancer cell proliferation in vitro. Collectively, these data indicate that the effects of Pten loss at heterozygote levels commonly observed in human tumours are modified by Igf2, and emphasise the importance of the evaluation of upstream pathways in tumours with Pten loss.

Materials and methods

Mice

Igf2tm1Rob (paternal allele disruption, Igf2+/y) H19tm11Ro (maternal allele disruption H19+/y) and Pten+/Re (heterozygote Pten+/+) mice were housed and genotyped by PCR as have been described previously (DeChiara et al., 1991; Leighton et al., 1995; Podsypanina et al., 1999) (see Supplementary Information for details of breeding and primer sequences). All
procedures were approved by the UK Home Office and performed under a Home Office Project license following the University of Oxford ethical committee approval.

**Tissue analysis**

RT-qPCR, immunoblotting, histology and immunohistochemistry were performed by standard techniques. Full methods are provided in Supplementary Information.

**3D Magnetic resonance microscopy**

Formalin-fixed foetuses obtained at E15.5 were suspended in phosphate-buffered saline doped with gadolinium diethylenetriaminepentaacetic acid (DTPA) and imaged by high-resolution magnetic resonance imaging microscopy by DJS Cardiac. Measurements were made using ImageJ (image processing and analysis in Java, http://rsbweb.nih.gov/ij/ (NIH)). Full details are provided in Supplementary information.

**Histopathology**

Haematoxylin and eosin-stained sections of mammary, endometrial and prostate tumours were analysed blinded to genotype by two experienced histopathologists (BRP and SM). Grading of neoplastic change was made according to published criteria (see Supplementary Information).

**miRNA analysis of embryos**

RNA was extracted from embryos at E9.5 and hybridised to Illumina mouse microRNA assay pool of 380 mouse microRNAs from Sanger miRBase v9.1 (http://www.mirbase.org/) as described in Supplementary Information (see also http://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-873).

**Cell culture**

MCF7 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% foetal calf serum, 1% glutamine, penicillin and streptomycin in a humidified atmosphere with 5% CO2 concentration. Stable expression of PTEN shRNA or a scrambled control sequence in MCF7 cells was achieved by standard retroviral methods as using the P-SuperRetro Puro vector (Oligoengine Seattle, WA, USA), and Phoenix packaging cells (Gentaur Europe, Ka menhout, Belgium, described fully in Supplementary Informa-

**Conflict of interest**

The authors declare no conflict of interest.

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