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ORIGINAL ARTICLE

Analysis of *Brca1*-deficient mouse mammary glands reveals reciprocal regulation of *Brca1* and *c-kit*

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Disruption of the breast cancer susceptibility gene *Brca1* results in defective lobular-alveolar development in the mammary gland and a predisposition to breast tumourigenesis in humans and in mice. Recent evidence suggests that *BRCA1* loss in humans is associated with an expansion of the luminal progenitor cell compartment in the normal breast and tumours with a luminal progenitor-like expression profile. To further investigate the role of *BRCA1* in the mammary gland, we examined the consequences of *Brca1* loss in mouse mammary epithelial cells *in vitro* and *in vivo*. Here, we show that *Brca1* loss is associated with defective morphogenesis of SCp2 and HC11 mouse mammary epithelial cell lines and that in the *MMTV-Cre Brca1^{Co/Co}* mouse model of *Brca1* loss, there is an accumulation of luminal progenitor (CD61$^+$CD29^{lo}CD24$^+$) cells during pregnancy. By day 1 of lactation, there are marked differences in the expression of 1379 genes, with most significantly altered pathways and networks, including lactation, the immune response and cancer. One of the most differentially expressed genes was the luminal progenitor marker, *c-kit*. Immunohistochemical analysis revealed that the increase in *c-kit* levels is associated with an increase in c-kit positivity. Interestingly, an inverse association between *Brca1* and *c-kit* expression was also observed during mammary epithelial differentiation, and small interfering RNA-mediated knockdown of *Brca1* resulted in a significant increase in *c-kit* mRNA levels. We found no evidence that *c-kit* plays a direct role in regulating differentiation of HC11 cells, suggesting that *Brca1*-mediated induction of *c-kit* probably contributes to *Brca1*-associated tumourigenesis via another cellular process, and that *c-kit* is likely to be a marker rather than a mediator of defective lobular-alveolar development resulting from *Brca1* loss.

Introduction

The mammary gland comprises a heterogeneous collection of epithelial cells that, in response to hormones and through interactions with stromal elements, undergo dynamic changes during the lactational cycle (reviewed in LaMarca and Rosen, 2008). Defining the molecular and cellular basis of this process is essential for understanding mammary development and associated defects, including breast cancer. The fact that breast cancer stem cells share some characteristics with mammary stem cells (Al-Hajj *et al.*, 2003; Visvader and Lindeman, 2008) and that mutations in a number of the genes associated with mammary development have also been implicated in breast cancer (Usary *et al.*, 2004; Bogorad *et al.*, 2008) suggests that studies on mammary development may help define the early events that precede breast tumourigenesis.

Experimental animal models have been invaluable in mammary gland research (reviewed in Hennighausen and Robinson, 2001), enabling the identification of mammary stem (Smalley *et al.*, 2005; Shackleton *et al.*, 2006; Stingl *et al.*, 2006); and luminal progenitor cells (Sleeman *et al.*, 2006, 2007; Asselin-Labat *et al.*, 2007) and the molecular changes that are associated with the different phases of post-pubertal mammary development (Master *et al.*, 2002; Clarkson *et al.*, 2004; Stein *et al.*, 2004). Mouse models displaying defects in these processes have also been very useful, for example, *Gata-3*, *Elf-5* and *Stat5a* knockout mice have helped define the cellular events involved in the development of luminal and secretory cells, respectively (Asselin-Labat *et al.*, 2007; Oakes *et al.*, 2008; Yamaji *et al.*, 2009), whereas *PrlR* and *Gal* knockout mice have shed light on

the molecular events associated with the secretory activation stage of lactation (Naylor *et al.*, 2005).

Disruption of the breast cancer susceptibility gene *BRCA1* results in defects in mammary epithelial differentiation *in vitro* (Kubista *et al.*, 2002; Furuta *et al.*, 2005) and defective lobular-alveolar development in the mammary gland *in vivo*, based on analysis of breast tissue from *BRCA1* mutation carriers or mice in which the *Brca1* gene has been deleted from the mammary gland or in which the mammary gland expresses a dominant-negative *Brca1* mutant (Jernstrom *et al.*, 1998; Xu *et al.*, 1999; Russo *et al.*, 2001; Brown *et al.*, 2002; Triplett *et al.*, 2008). Tumours arising in *BRCA1* mutation carriers and *Brca1* conditional knock-out mice have a distinct histopathological and molecular phenotype, including high grade, high mitotic index and expression of basal and stem cell-associated genes (Lakhani *et al.*, 1998; Sorlie *et al.*, 2003; Liu *et al.*, 2008; Shakya *et al.*, 2008; Wright *et al.*, 2008a, b). It has been suggested that *BRCA1*-associated tumours arise in the basal or stem cell compartments of the mammary gland (Foulkes *et al.*, 2003), although there is also evidence to suggest that that these characteristics may arise as a consequence of *BRCA1* loss in other mammary cell types (Liu *et al.*, 2008; Smart *et al.*, 2008). Although the detailed pathway between *BRCA1* disruption and breast tumour development has yet to be fully elucidated, recent findings suggest that tumours arising in human *BRCA1* mutation carriers may arise from mammary luminal progenitor cells of the mammary gland (Lim *et al.*, 2009).

To further investigate the role of *BRCA1* in mammary development and cancer, we examined the cellular and molecular consequences of mouse *Brca1* loss *in vitro* and *in vivo*. Here, we show that loss of *Brca1* results in a defect in mammary epithelial morphogenesis and lobular-alveolar development that is associated with an accumulation of luminal progenitor cells and differential expression of multiple genes, including *c-kit*. Functional studies on *c-kit* suggest that, although it can be regulated by *Brca1*, it is unlikely to be responsible for the *Brca1*-associated defect in lobular-alveolar development, and thus is more likely to be a marker than a mediator of the phenotype of these mice.

Results

Accumulation of luminal progenitor cells in the mammary glands of MMTV-Cre Brca1$^{Co/Co}$ mice during pregnancy
Brca1 has been shown to play an important role in mouse mammary epithelial differentiation *in vitro* (Kubista *et al.*, 2002). To confirm and extend previous studies, we examined the consequences of *Brca1* suppression in two mouse mammary epithelial cell lines HC11 and SCp2, and examined the morphological and cellular changes in the mammary glands of *MMTV-Cre Brca1$^{Co/Co}$* mice. As shown in Figures 1a and b, repression of *Brca1* in both cell lines caused a defect in mammosphere formation. Histological analysis of SCp2

mammospheres further revealed disorganised acini-like structures, relative to controls (Figure 1b). Consistent with this and with previous reports, mammary-specific depletion of *Brca1 in vivo* resulted in defective lobular-alveolar development, characterised by distended lumen and disorganised alveoli (Xu *et al.*, 1999; Brown *et al.*, 2002; Figure 1c).

Although the cellular basis of the *in vivo* phenotype is unknown, the observed expansion of the luminal progenitor cell population in the breast tissue of human *BRCA1* mutation carriers (Lim *et al.*, 2009) led us to hypothesise that there may be an accumulation of luminal progenitor cells in the mammary glands of *MMTV-Cre Brca1$^{Co/Co}$* mice. We therefore examined CD61$^+$CD29^{lo}CD24$^+$ luminal progenitor cells isolated from virgin and pregnant mammary glands of *MMTV-Cre Brca1$^{Co/Co}$* and control mice. These time points were selected, as the luminal progenitor (CD61$^+$) population declines dramatically during pregnancy and is virtually undetectable in late pregnancy and lactation. No significant difference in the number of CD61$^+$CD29^{lo}CD24$^+$ progenitor cells was observed in the resting virgin mammary glands of *MMTV-Cre Brca1$^{Co/Co}$* mice compared with controls (Figure 1d, left bars). During pregnancy, however, when luminal progenitors are induced to differentiate and transgene activity is augmented, we found that the proportion of CD61$^+$CD29^{lo}CD24$^+$ cells was significantly increased in *MMTV-Cre Brca1$^{Co/Co}$* mice, relative to littermate controls (Figure 1d, right bars).

Significant changes in the expression profile of mammary glands from MMTV-Cre Brca1$^{Co/Co}$ mice at day 1 of lactation associated with Brca1 deficiency
To begin to investigate the molecular basis of this observation, gene expression profiling was performed on three pools of four mammary glands from *MMTV-Cre Brca1$^{Co/Co}$* and control *Brca1$^{Co/Co}$* mice at 10 weeks of age (virgin) and day 1 of lactation (Figure 2a), for which *Brca1* recombination and depleted expression was verified.

The choice of the day 1 lactation time point was based on the time at which the mammary phenotype was most evident in *MMTV-Cre Brca1$^{Co/Co}$* mice, and that it is technically easier to precisely identify mice at day 1 of lactation rather than a specific day in pregnancy. Such precision is essential for molecular profiling studies, given the dynamic molecular changes occurring in the mammary gland during lactational development. *Brca1$^{Co/Co}$* mice were used as a control to ensure that our data were not confounded by effects of the *Brca1$^{Co/Co}$* locus on the expression of *Brca1* splice isoforms. The lactational phenotype is not observed in *MMTV-Cre* mice (Supplementary Figure 4), indicating that this phenotype is unlikely to be a result of Cre recombinase expression only in the gland.

A robust statistical analysis of microarray data (see Materials and methods) identified 1379 probes that were differentially expressed between *MMTV-Cre Brca1$^{Co/Co}$* and *Brca1$^{Co/Co}$* mice at day 1 of lactation (Supplementary Table 1). Of these, 786 were upregulated (44 with a greater than twofold change) and 593 were downregulated
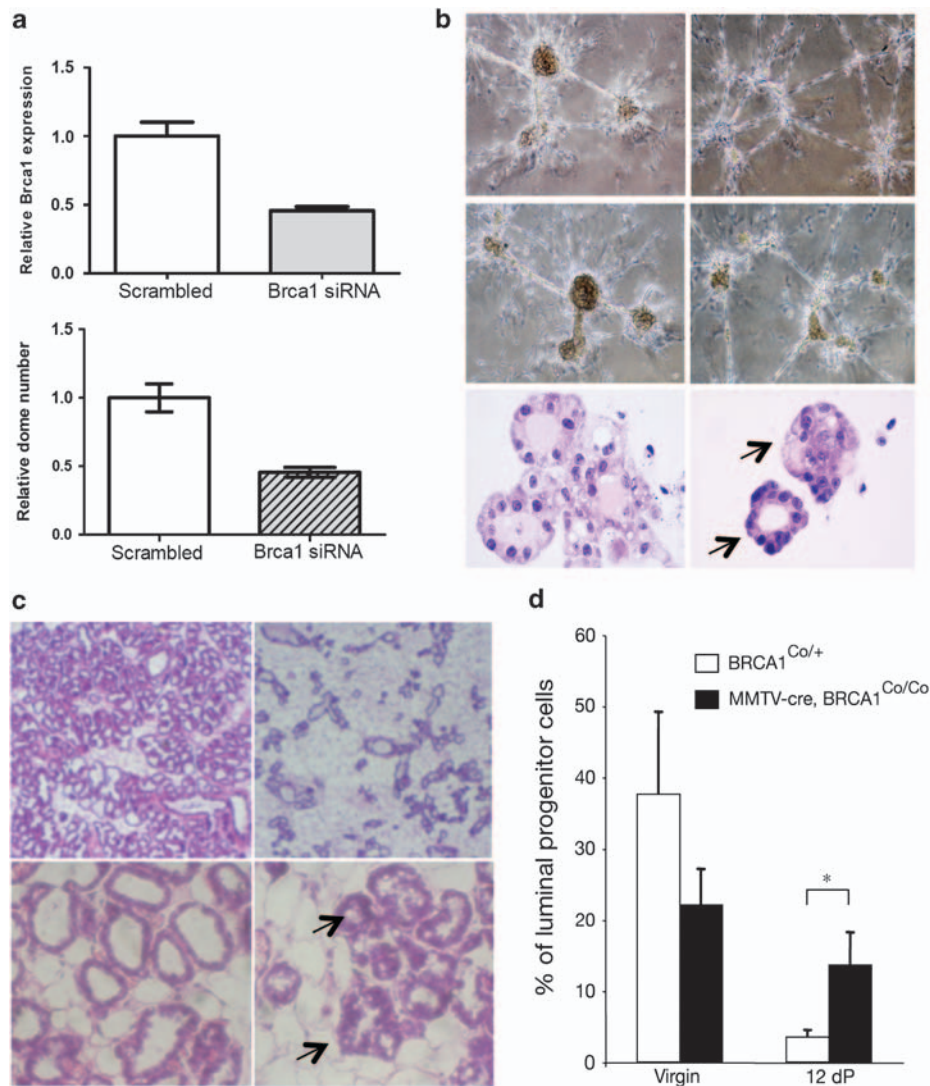
Figure 1 Suppression of *Brca1* results in defective mammary epithelial morphogenesis *in vitro* and accumulation of luminal progenitor cells *in vivo*. (**a**) *Brca1* knockdown by siRNA is confirmed in HC11 cells by real-time PCR (top graph). *Brca1* mRNA levels were compared in HC11 cells transfected with scrambled (control) and *Brca1* siRNA constructs. Bars represent the mean of three independent experiments and error bars represent s.e. Knockdown of *Brca1* reduces dome formation in the HC11 *in vitro* differentiation assay (bottom panel). Dome formation was calculated at day 8 after initiation of the assay and shown here relative to the scrambled control. Error bars represent s.e. (**b**) Functional disruption of Brca1 impairs prolactin-induced mammary differentiation of SCp2 cells. Differentiation of SCp2 cells transfected with *pZeoSV* vector (left column) and *trBrca1* (right column). Phase contrast images ($\times 20$ magnification) are shown of SCp2 cells that were grown on matrigel, four days (top row) and six days (middle row) after the addition of lactogenic hormones. H&E-stained sections (bottom row) of cell aggregates at day 6 were photographed using a light microscope at $\times 200$ magnification. Arrows indicate defective morphogenesis. (**c**) H&E-stained sections of day 1 lactation mammary glands taken from *Brca1^{Co/Co}* (left column) and *MMTV-Cre Brca1^{Co/Co}* (right column) mice taken at $\times 40$ (top) and $\times 100$ (bottom) magnifications. Arrows indicate defective morphogenesis. (**d**) Sorted mammary epithelial cells from *MMTV-Cre Brca1^{Co/Co}* mice exhibit increased luminal progenitor cells at day 12 of pregnancy. Histograms showing percentage of CD61$^+$ cells in the CD29^{lo}CD24$^+$ population isolated from virgin or 12.5-day pregnant *Brca1^{Co/+}* and *MMTV-Cre, Brca1^{Co/Co}* mammary glands (mean ± s.e.m. of 3–6 animals per group). *$P < 0.05$ (Student's *t*-test).

(3 with greater than twofold change) in *MMTV-Cre Brca1^{Co/Co}* mice. In contrast, only 38 genes were differentially expressed in the 10-week old virgin mice (Supplementary Table 2).

Validation of multiple differentially expressed genes, including c-kit
To validate individual genes corresponding to the probes identified in the microarray data, quantitative real-time

PCR analysis of selected genes was performed, using RNA isolated from 8 of the 12 individual mice that comprised the pools used in the array analysis and normalising to hypoxanthine phosphoribosyltransferase 1 (HPRT). The genes selected for validation spanned a range of fold changes and B-scores, and were components of functional categories that Ingenuity pathway analysis indicated were significantly overrepresented in our gene list. The relative gene changes seen using quantitative real-time PCR and the fold changes given by the microarray analysis were
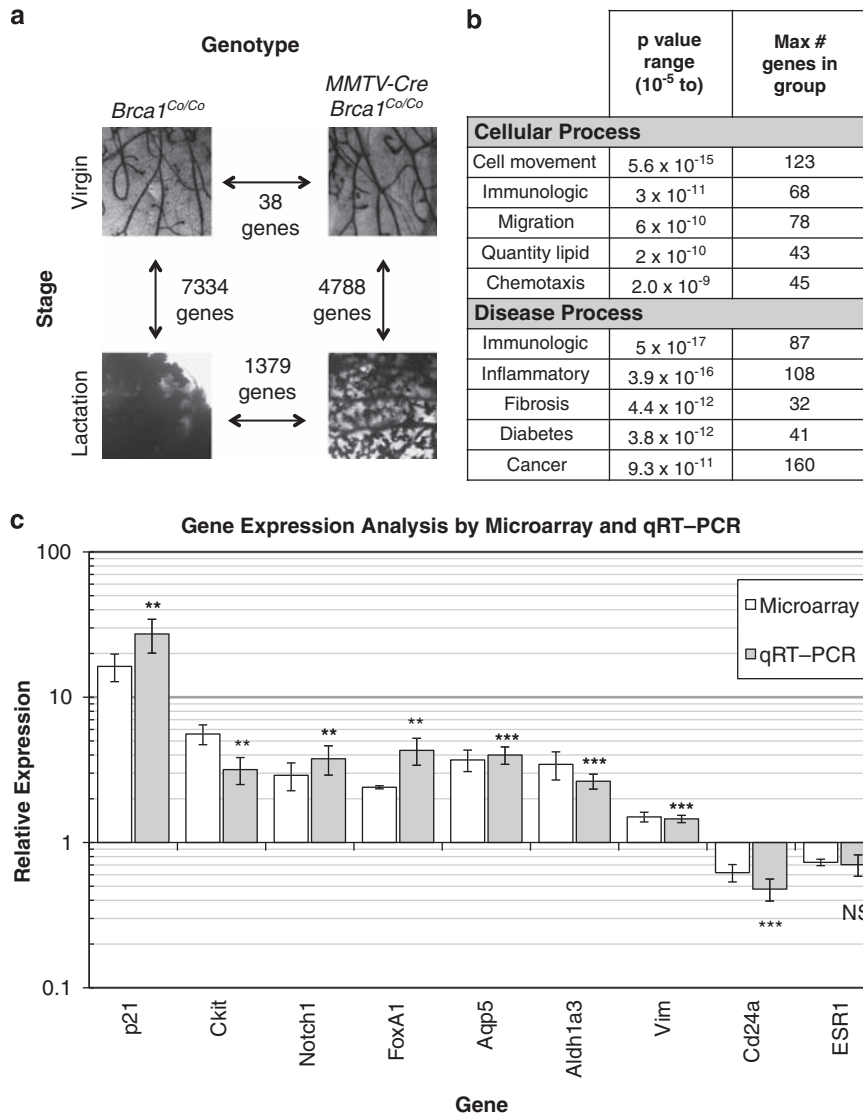
Figure 2 Differential expression of genes in the mammary glands of *MMTV-Cre Brca1$^{Co/Co}$* mice at day 1 of lactation. (**a**) Experimental design. Gene expression profile analysis was performed on RNA extracted from the mammary glands of *Brca1$^{Co/Co}$* and *MMTV-Cre Brca1$^{Co/Co}$* mice at 10 weeks of age (virgin) and day 1 of lactation. Three pools of four individual mice were used to represent each stage and genotype. Data analysis revealed the indicated number of differentially expressed genes. (**b**) Results of ingenuity analysis showing the most significantly altered cellular and disease processes in *MMTV-Cre Brca1$^{Co/Co}$* mice. (**c**) Comparison of gene expression data in day 1 lactation *MMTV-Cre Brca1$^{Co/Co}$* mice compared with controls by microarray (fold change (white bars)) and validating quantitative real-time PCR (relative expression (grey bars)) of eight independently analysed individual mice. Error bars on microarray data represent s.e. of the three pools and on real-time PCR data represent the s.e.m. of eight biological replicates. Statistical analysis was performed using a Student's *t*-test with ***$P < 0.001$ and **$0.001 < P < 0.01$. NS, not significant; qRT–PCR, quantitative real-time PCR.

similar, demonstrating the robustness of the microarray data (Figure 2c).

The highest B-score that validated was 16.3 (*p21*) and the lowest B-score that validated was 1.19 (*CD24a*), a range of B-scores attributed to over 500 transcripts (Supplementary Table 1). Quantitative real-time PCR was also used to confirm that *Brca1* transcript levels were significantly reduced in the mammary glands of *MMTV-Cre Brca1$^{Co/Co}$* compared with control *Brca1$^{Co/Co}$* mice (data not shown), further validating the experimental design and expression data.

Given the fold increase of *c-kit* observed in *MMTV-Cre Brca1$^{Co/Co}$* glands, together with its likely importance

in *Brca1*-associated tumourigenesis in humans (Nielsen *et al.*, 2004; Lim *et al.*, 2009), our initial studies focused on the expression, regulation and function of *c-kit* in mammary epithelial cells.

An increase in c-kit immunopositivity in lactating mammary glands of MMTV-Cre Brca1$^{Co/Co}$ mice at day 1 of lactation

As the expression profiling experiments were performed using RNA from whole-mammary glands, the observed changes in gene expression may reflect molecular events in any of the cellular compartments of the mammary

gland. To determine where c-kit protein is normally expressed in the lactating mammary gland and the spatial consequences of *Brca1* loss, immunofluorescence analysis of c-kit was performed on frozen sections of optimal cutting temperature (OCT) media-embedded glands of *MMTV-Cre Brca1*$^{Co/Co}$ ($n = 6$) and *Brca1*$^{Co/Co}$ ($n = 4$) mice at day 1 of lactation. Control *Brca1*$^{Co/Co}$ mice displayed a distinct lack of c-kit expression, with only sporadic staining in the luminal epithelium of the mammary ducts (Figure 3a). In contrast, the mammary glands of *MMTV-Cre Brca1*$^{Co/Co}$ mice demonstrated heterogeneous c-kit immunostaining throughout the mammary epithelium (Figure 3a). Some c-kit immunopositivity was observed in the interstitium of mice of both genotypes, perhaps reflecting the presence of mast cells.

Reciprocal regulation of Brca1 and c-kit in mammary epithelial cells

Although expression profiling and immunohistochemical analysis provides important information on the consequences of *Brca1* loss, it does not allow conclusions to be made about the molecular relationship between *Brca1* and the differentially expressed genes including *c-k*it within a given cell. To address this, experiments were performed using the mouse mammary epithelial cell line HC11. In the first instance, the endogenous expression of *Brca1* and *c-kit* was determined during differentiation using real-time PCR. As shown in Figure 4a, *Brca1* expression levels were reduced by ∼threefold as the cells underwent differentiation. In contrast, the levels of *c-kit* were induced by ∼200-fold. This result raises the possibility that

Brca1 and *c-kit* are coordinately, albeit reciprocally regulated.

To address the hypothesis that the increase in *c-kit* expression is a consequence of *Brca1* repression in mammary epithelial cells, *Brca1* levels were repressed using small interfering RNA (siRNA). As shown in Figure 4b, repression of *Brca1* was associated with an increase in the levels of *c-kit* mRNA. These data indicate that induction of *c-kit* may be a consequence of *Brca1* repression in the epithelial compartment of the mammary gland.

c-kit does not regulate HC11 mammary epithelial differentiation

Induction of *c-kit* as a consequence of *Brca1* repression in mammary epithelial cells suggests that *c-kit* may be a mediator of some of the effects of *Brca1* loss, including the observed defects in mammary epithelial differentiation. To begin to address this hypothesis, the effect of perturbing *c-kit* expression on HC11 differentiation was determined. Overexpression, achieved by retroviral transduction and confirmed by real-time PCR, had no effect on the number of HC11 domes formed (Figure 5a) or the expression of β-casein, a marker of terminal differentiation (data not shown). Similarly, knockdown of expression, achieved by siRNA and confirmed by real-time PCR, similarly had no significant effect on the number of HC11 domes (Figure 5b) or the expression of β-*casein* (data not shown). These data indicate that *c-kit* does not regulate the differentiation of HC11 mammary epithelial cells. However, this does not preclude a physiological role for *c-kit* in the mouse mammary gland.
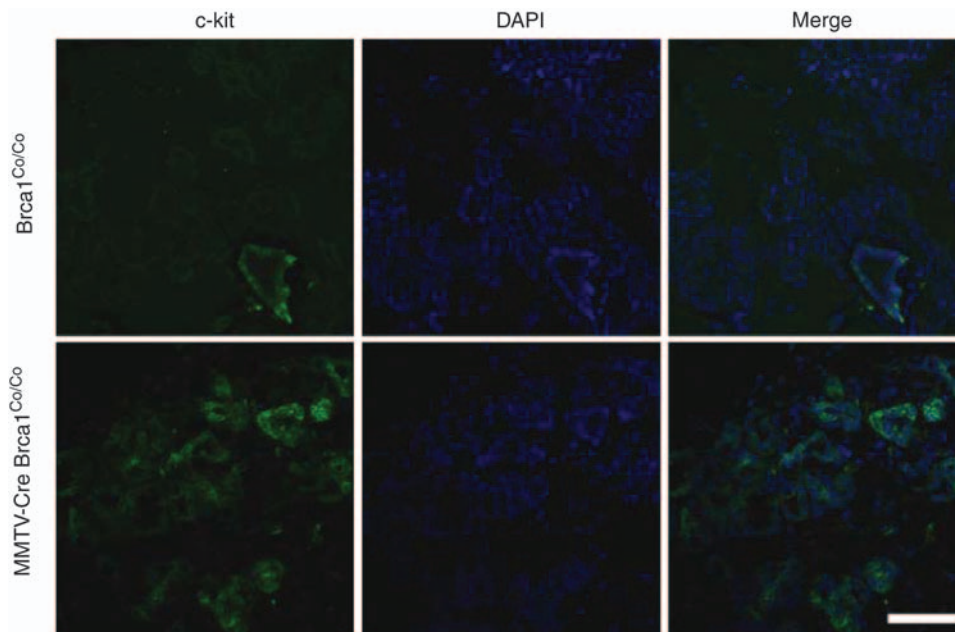

Figure 3   An increase in c-kit immunopositivity in the mammary glands of *MMTV-Cre Brca1*$^{Co/Co}$ mice. Immunofluorescence analysis of c-kit (green) and DAPI (blue) nuclear counterstain in frozen sections of day 1 lactating mammary glands. Images represent glands from *Brca1*$^{Co/Co}$ mice and *MMTV-Cre Brca1*$^{Co/Co}$ mice. Images were taken at ×200 magnification. Scale bars represent 100 μm.
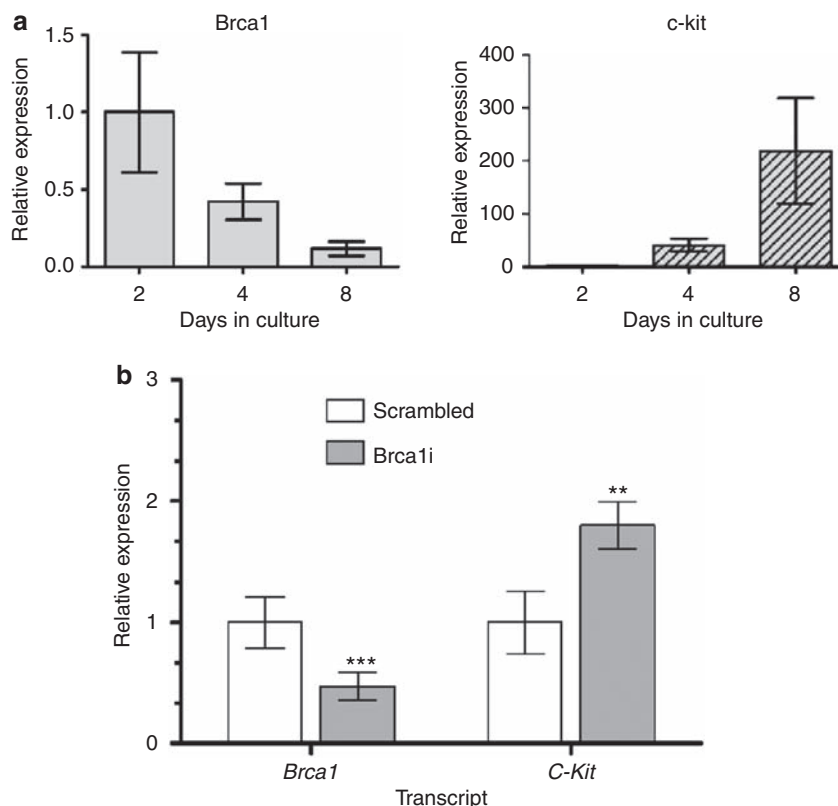
Figure 4 Reciprocal regulation of *Brca1* and *c-kit* in mammary epithelial cells. (**a**) Endogenous *Brca1* and *c-kit* mRNA expression was measured in HC11 cells throughout 8-day *in vitro* differentiation with lactogenic stimulus. Bars represent the mean of three independent experiments and error bars represent s.e., and significance was shown to be $P < 0.05$ between days 2 and 4 and < 0.005 between days 2 and 8, as determined by Student's *t*-test. (**b**) *Brca1* knockdown in HC11 cells was achieved with transfection of *Brca1* siRNA (grey bars) and compared with scrambled controls (white bars). Knockdown of *Brca1* in proliferation was confirmed by quantitative PCR of the *Brca1* mRNA and the effect on *c-kit* mRNA was also quantified. Expression is shown relative to HPRT, and bars represent the mean of three independent experiments. Error bars represent s.e., and significance was determined by Student's *t*-test with ***$P < 0.001$, **$0.001 < P < 0.01$.

Discussion

Disruption of the breast cancer susceptibility gene *BRCA1* results in defective lobular-alveolar development of the mammary gland and predisposes to breast tumourigenesis, but the molecular and cellular basis of this phenotype is not well understood and has been investigated in this manuscript. Here, we show that loss of *Brca1* results in a defect in mammary epithelial morphogenesis that is associated with an accumulation of luminal progenitor cells and with a significant number of gene expression changes.

It is interesting that different lines of *MMTV-Cre* transgenic mice sometimes display different morphological phenotypes, including defective lobular-alveolar development (Cui *et al.*, 2002; Pylayeva *et al.*, 2009); however, this is usually attributable to the well-documented differences in spatial and temporal expression of the *MMTV-Cre* transgene and thus recombination-mediated deletion of the target gene (Wagner *et al.*, 2001), rather than to effects of the Cre recombinase. In the case of *Brca1*, defective lobular-alveolar development is consistently observed using two independent MMTV-Cre lines: line F (Figure 1a) and line A

(Triplett *et al.*, 2008), and *WAP-Cre Brca1*[Ko/Co] mice (Xu *et al.*, 1999; Triplett *et al.*, 2008), arguing strongly that the observed mammary phenotypes are a consequence of *Brca1* loss. This is further supported by our supplementary data showing that the mammary glands of *MMTV-Cre* mice at day 1 of lactation are indistinguishable from wild-type control mice (Supplementary Figure 4). In the case of more subtle molecular changes, however, we cannot completely exclude a potential contribution of *MMTV-Cre* on the expression of some genes. This is one of the many limitations of Cre-loxP technology (reviewed in Maddison and Clarke, 2005).

Our data suggest that a differentiation block exists in luminal progenitor cells of the mammary glands of *MMTV-Cre Brca1*[Co/Co] mice. These observations support the finding that *Brca1*-deficient ER-negative primary mammary progenitor cells are defective in their ability to differentiate into ER-positive luminal cells *in vitro* (Liu *et al.*, 2008) and that aberrant luminal progenitor cells are expanded in breast tissue from human *BRCA1* mutation carriers (Lim *et al.*, 2009). Defects in luminal progenitor cells have been observed in other mouse models, including *MMTV-Cre Gata-3*^{fl/+}
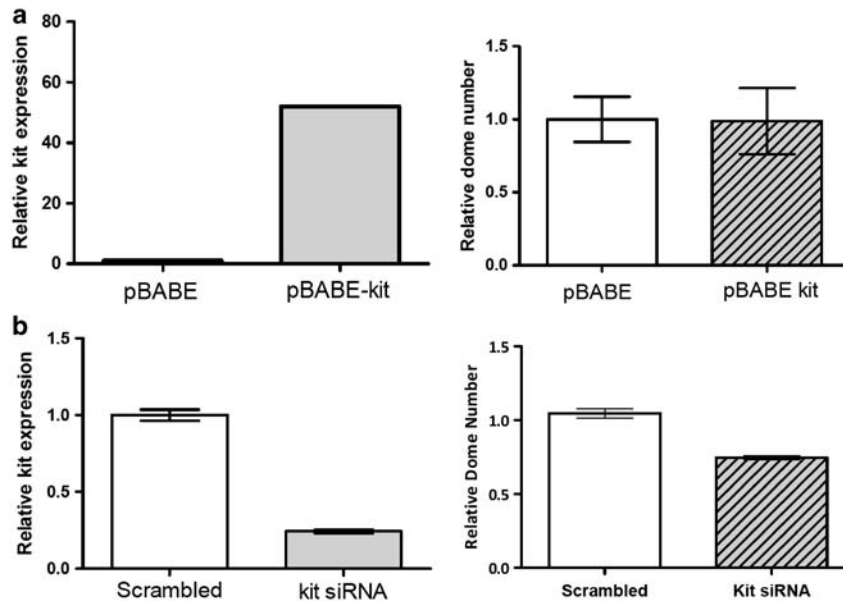
Figure 5 c-kit does not regulate HC11 differentiation. Ectopic *c-kit* expression (**a**) and knockdown (**b**) was confirmed in HC11 cells by real-time PCR (left-hand graphs). *c-kit* mRNA levels were compared in HC11 cells transfected with (**a**) pBABE (control) and pBABE-c-kit or with (**b**) scrambled (control) and *c-kit* siRNA constructs. Bars represent the mean of three independent experiments (relative HPRT) and error bars represent s.e. Dome formation was unaffected by ectopic *c-kit* expression but was reduced with *c-kit* knockdown (right-hand graphs). Dome formation was calculated at 8 days after initiation of the assay and shown here relative to the pBABE (**a**) or scrambled (**b**) controls. Error bars represent s.e.

mice, which exhibit an expansion of the $CD61^+$ progenitor population during puberty and pregnancy (Asselin-Labat *et al.*, 2007), and $Elf5^{-/-}$ and $Stat5a^{-/-}$ mice, which show an increase in these progenitors at mid-pregnancy (Oakes *et al.*, 2008; Yamaji *et al.*, 2009). Interestingly, non-parous precancerous tissues from *BRCA1* mutation carriers harbour an aberrant luminal progenitor population (Lim *et al.*, 2009), whereas young virgin mice do not (Figure 1d). This is most likely to be due to differences in age and parity, although a more thorough analysis of this, including analysis of mice at different ages and after pregnancy cycles, will be necessary to firmly establish this.

Many of the observed molecular changes, including repression of milk protein genes, are consistent with previously reported expression changes in mammary glands and epithelial cell lines in which *Brca1* function has been diminished (Brown *et al.*, 2002; Furuta *et al.*, 2005) and in mammary glands lacking *Brca1*. However, a comparison of our gene list with the expression changes that occur during normal lactational development of the mouse mammary gland, and with other mouse models of lactational delay, indicate, however, that the molecular changes induced by loss of *Brca1* are not solely a reflection of the observed defect in lobular-alveolar development, and that many of the observed changes are likely to reflect other consequences of *Brca1* loss. These data, therefore, provide a resource for the identification and analysis of novel functions of *Brca1*, and for better understanding how *Brca1* contributes to normal development and disease.

Genes upregulated more than fivefold in the mammary glands of *MMTV-Cre Brca1^{Co/Co}* mice at day 1 of lactation include *Cdkn1A* (p21, up 16.3-fold), a cell cycle regulated that has previously been shown to be upregulated upon *Brca1* repression (Hakem *et al.*, 1996) and that has been implicated in breast cancer (Weiss, 2003); interferon-stimulated gene 15 (*Isg15*, up 10.9-fold) and the gene encoding the Isg15-interacting protein *Ubp43* (up eightfold), the former being a ubiquitin-like gene implicated in immune response to viruses and in breast cancer (Bektas *et al.*, 2008); oligoadenylate synthetase 1G (up 9.2-fold) and 1A (up 5.7-fold), an interferon-induced enzyme implicated in nucleotide metabolism and expressed in the mammary gland (Maia *et al.*, 2008) and induced upon irradiation of human breast cancer cell lines (Tsai *et al.*, 2007); and *c-kit* (up 5.58-fold), a hematopoietic stem cell marker that is overexpressed in undifferentiated mammary tumours (Tsuda *et al.*, 2005) and functions as an oncogene in mammary epithelial cells (Tsuda *et al.*, 2005; Raafat *et al.*, 2007). Taken together, these genes suggest a role for *Brca1* in the immune response and cancer.

The finding that multiple cancer-associated genes are overrepresented in the expression profile of *MMTV-Cre Brca1^{Co/Co}* mice is also interesting. This is in contrast to other mouse models with a mammary phenotype (Naylor *et al.*, 2005), Ingenuity pathway analysis has identified multiple functional themes with statistical significance greater than those associated with cancer (data not shown). This provides additional evidence that the observed changes in gene expression in *MMTV-Cre Brca1^{Co/Co}* mice are consequences of *Brca1* loss rather than those associated with the mammary phenotype. It is also raises the possibility that some of the observed

changes also represent early changes in _Brca1_-associated tumourigenesis.

One of the most significantly overrepresented genes (up 5.58-fold), participating in the cancer network, is _c-kit_ (Figure 2c). _c-kit_ has recently been identified as a marker of luminal progenitor cells in the human breast (Lim _et al_., 2009). Although making comparisons across species and at different stages of lactational development must be done with caution, it is interesting to note the overrepresentation of _c-kit_ in the mammary glands of _MMTV-Cre Brca1_$^{Co/Co}$ mice at day 1 lactation is consistent with our fluorescence activated cell sorting (FACS) analyses showing an accumulation of luminal progenitor cells during pregnancy (Figure 1b). Although analysing c-kit expression through other developmental time points would facilitate comparisons between the molecular and cellular profiling experiments, these data do support the notion that luminal progenitor cell population is expanded in Brca1-deficient mammary glands. It would also be interesting to examine the luminal progenitor population in mammary glands after involution to determine how stable the observed cellular changes are.

To begin to investigate whether there is a molecular link between _Brca1_ and _c-kit_, we analysed the expression of both genes during mammary epithelial differentiation, and the consequences of _Brca1_ repression on _c-kit_ expression, _in vitro_. Our data suggest that there is a regulatory link between _Brca1_ and _c-kit_, albeit probably indirect. The _c-kit_ promoter and 3' UTR regions have been well characterised by others and many regulators, including the transcription factor Myb (Vandenbark _et al_., 1996) and the microRNA 221 (Felli _et al_., 2005) have been described. Future studies aimed at elucidating the molecular basis of _Brca1_-mediated regulation of _c-kit_ are currently underway.

Although _Brca1_ repression was clearly associated with the accumulation of luminal progenitor cells and increased _c-kit_ levels, and _Brca1_ in turn regulated the expression of _c-kit_ in mammary epithelial cells, it was unclear whether the observed overexpression of _c-kit_ was a bystander or mediator of the observed defect in lobular-alveolar development in _Brca1_-deficient mice. Experiments in HC11 cells in which _c-kit_ was overexpressed or knocked down indicated that _c-kit_ may not have a direct role in regulating epithelial differentiation, at least in these cells. There are at least two possible explanations for this result, first, it may be that a role for c-kit in mammary differentiation is p53 dependant and thus a phenotype would not be observed in p53 mutant cell lines such as HC11. Alternatively, the observed defect in lobular-alveolar development may be mediated by molecules other than c-kit, potentially including other differentially expressed genes identified in this study (Supplementary Table 1). This data further suggest that _Brca1_-mediated induction of _c-kit_ is more likely to contribute to other changes in the phenotype of luminal progenitor cells. c-kit mediated hyperproliferation of luminal progenitor cells is a reasonable hypothesis that is supported by the literature (Kijima _et al_., 2002) and the observed imbalance of these cells in the _Brca1_ deficient mammary gland (Figures 1 and 3). _c-kit_

induction may also induce other cellular changes. Studies by other investigators have shown that repression of _c-kit_ results in a decrease in anchorage-independent growth (Raafat _et al_., 2007), raising the possibility that _Brca1_ induction of _c-kit_ might contribute to the oncogenic transformation of luminal progenitor cells rather than a block in their differentiation. Consistent with this notion is the finding that luminal progenitor cells from human _BRCA1_ mutation carriers display factor-independent growth (Lim _et al_., 2009).

Overrepresentation of c-kit in _Brca1_-deficient mammary glands is also consistent with the finding that c-kit is overexpressed in a proportion of human and mouse _Brca1_-associated mammary tumours (Herschkowitz _et al_., 2007; Lim _et al_., 2009). This supports our previous hypothesis that luminal progenitor cells, rather than basal or stem cells, are the target of _BRCA1_-associated tumourigenesis (Lim _et al_., 2009), which in turn is consistent with our previous studies showing no mammary phenotype when _Brca1_ is specifically deleted in non-luminal compartments of the mammary gland (Smart _et al_., 2008).

In summary, we show that loss of _Brca1_ in mouse mammary epithelial cells results in a block in differentiation and an accumulation of luminal progenitor cells. This is associated with overexpression of _c-kit_, a marker of luminal progenitor cells. Based on _in vitro_ experiments, c-kit may simply serve as a cell marker rather than a mediator of the observed defect in lobular-alveolar development. However, analysis of _c-kit_ mutant mice will be required to further address the role of c-kit, and may reveal a role in regulating commitment to the luminal cell lineage. Future priorities will also include defining the role of _c-kit_ in _Brca1_-associated tumourigenesis and establishing whether targeting _c-kit_ before tumour formation can interfere with this process.

Materials and methods

HC11 and SCp2 mammary epithelial morphogenesis assays
HC11 cells were supplied by Chris Ormandy (Garvan Institute, Sydney, NSW, Australia) and cultured as previously described (Naylor _et al_., 2005). _Brca1_ repression was achieved by _Brca1_ siRNA (L-040545-00) and non-targeting (D-001810-10-05) purchased from Dharmacon RNA Technologies (Lafayette, CO, USA), and used at 100 nM. HC11 cells were transfected with lipofectamine 2000 as described in Naylor _et al_. (2005) and incubated for 24 h. Knockdown was confirmed by real-time PCR, as described below. HC11 differentiation assays were performed as previously described (Naylor _et al_., 2005). SCp2 cells were supplied by Mina Bissell (Lawrence Berkeley National Laboratory, Berkeley, CA, USA). Repression of Brca1 function was achieved by ectopic expression of a truncated (dominant-negative) mouse _Brca1_ as described previously (Brown _et al_., 2002). Six independent pools of 7×10^5 _trBrca1_-expressing or control SCp2 cells were plated on Engelbreth-Holm-Swarm-derived extracellular matrix (Matrigel, Becton Dickinson, Franklin Lakes, NJ, USA) in growth media containing reduced fetal calf serum (FCS) (2%). Cells were allowed to attach for 24 h and then stimulated to differentiate in serum-free media containing 3–5 µg/ml ovine prolactin (AF Parlow, Los Angeles, UCLA, CA, USA), 1 µg/ml hydrocortisone and 5–10 µg/ml insulin. SCp2 cells were fixed in neutral-buffered formalin (Sigma-Aldrich, St Louis, MO, USA)

and processed to paraffin wax. Sections were analysed by staining with haematoxylin and eosin.

Mice
MMTV-Cre (Wagner *et al.*, 1997) and *Brca1$^{Co/Co}$* mice (Xu *et al.*, 1999) were obtained from the NCI, Frederick, MD, USA. F1 and F2 matings, and genotyping were performed as described (Xu *et al.*, 1999). The *MMTV-Cre* mice used were transgenic line F (NCI catalogue #01XA9). To minimise the effects of expression changes associated with stage of the oestrus cycle, multiple independent groups of six mice were used from the same female-only cage, and the vulva of each mouse was checked to ensure comparable vascularisation. All procedures were approved by The University of Queensland Animal Ethics Committee. Mammary gland dissections for RNA and whole-mount analysis were performed as previously described (Smart *et al.*, 2008).

Mammary luminal progenitor cell analysis
The thoracic and inguinal mammary glands were dissected from virgin or 12.5-day pregnant *Brca1$^{Co/+}$ and MMTV-Cre Brca1$^{Co/Co}$* female mice. Mammary epithelial cell suspensions were prepared as previously described (Shackleton *et al.*, 2006; Asselin-Labat *et al.*, 2007). Cells were stained with anti-CD31 and anti-CD45 antibodies conjugated to biotin (BD Pharmingen, Franklin Lakes, NJ, USA), followed by streptavidin-allophycocyanin (APC)-Cy7 (BD Pharmingen) or streptavidin-Alexa-594 (Molecular Probes, Invitrogen, Carlsbad, CA, USA). Anti-CD24 conjugated to phycoerythrin (PE) (BD Pharmingen), anti-CD29 conjugated to fluorescein isothiocyanate (clone HMβ1.1 kindly provided by Dr H Yagita, Juntendo University, Tokyo, Japan) and anti-CD61 conjugated to APC (Caltag, Invitrogen) were used. Cell sorting and analysis was carried out on a FACS ARIA or FACSDiVa (Becton Dickinson).

RNA extraction and gene expression analysis
RNA was extracted from the abdominal mammary glands (after removal of lymph nodes) of 12 mice per genotype and stage (virgin and lactation). One entire abdominal mammary gland for each mouse was ground on dry ice for RNA extraction using an RNAeasy Extraction kit (Qiagen, GmbH, Hilden, Germany) according to the manufacturers' instructions. The RNA quantity and integrity was assessed with a Bioanalyser (Agilent Technologies, Forest Hill, VIC, USA). Three pools of four individual mice per genotype and stage were then generated, creating a total of 12 pools. The Illumina Totalprep RNA amplification kit (Applied Biosystems, Foster City, CA, USA) was used to amplify and biotinylate the 12 pooled RNA samples. Biotinylated RNA was hybridised overnight at 55 °C to Illumina Mouse Sentrix 6 v1.1 BeadChips containing > 46 000 probes (Illumina Inc., San Diego, CA, USA). The microarrays were washed, stained with streptavidin-Cy3, and then scanned with an Illumina BeadArray Scanner as per manufacturer's guidelines. All microarray data is available for download from the NCBI at GEO (Accession number: pending). Raw data was imported into Illumina Beadstudio v2.6, then further analysed with Genespring v7.3 (Agilent Technologies) and R Bioconductor packages BeadArray (Dunning *et al.*, 2007) and Limma (Smyth *et al.*, 2005). Data were normalised with quantile normalisation and filtered using an Illumina detection score of 0.95, yielding 23 891 probes. Unsupervised cluster analysis was performed within the GeneSpring package (Agilent, Santa Clara, CA, USA) using the Pearson's correlation for the distance metric on genes that differed two-fold from the mean in at least one sample. Genes differentially expressed between genotype, or stage, were determined using the Limma package (WEHI Bioinformatics,

Melbourne, VIC, Australia) in 'R'. A total of 1379 differentially expressed genes (B-score > 0) at the lactational stage were examined by Ingenuity Pathway Analysis Systems (www.ingenuity.com). Overrepresented biological themes were scored by significance. A threshold was determined by inputting the same number of random Illumina Mouse Sentrix 6 v1.1 probe codes as in the microarray results in triplicate and using the highest $-\log(P\text{-value})$ as the background level of significance.

Real-time PCR
Primers were designed using Primer Express (Applied Biosystems) and supplied by Geneworks (Australia, Hindmarsh, SA, Australia) (Supplementary Table 4). Analysis of complementary DNA (cDNA) from virgin and lactating glands was performed using SYBR Green Master Mix (Applied Biosystems) and parous glands using the FastStart Universal SYBR Green Master Mix (Roche Applied Sciences, Castle Hill, NSW, Australia). Gene expression was quantified on an ABI 7900HT Fast Real-time PCR system with ABI 7900HT SDS software (v3.3.3, Applied Biosystems), normalising to HPRT. Cycling conditions were 2 min (50 °C), followed by 10 min (95 °C) and then onto 45 cycles of 15 s (95 °C) and 1 min (58 °C). The comparative ΔCt method (Applied Biosystems) was used to determine relative mRNA expression (Livak and Schmittgen, 2001). The values were then further normalised to the wild-type average to ascertain the expression difference between genotypes. Statistical analysis used a two-tailed Student's *t*-test. A *P*-value of 0.05 or less was considered statistically significant.

Immunofluorescence analysis
In all, 7 μm sections of OCT-embedded mammary glands were fixed with 100% cold acetone. Sections were blocked in 5% fetal calf serum, 1% bovine serum albumin and 0.1% Tween-20 in phosphate buffered saline for 30 min and then incubated with 1:100 dilution of rat anti-murine c-kit antibody (Cedarlane, Burlington, Ontario, Canada) in the same blocking buffer for 1 h at room temperature. Goat anti-rat Alexa-Fluor 488 (Molecular Probes) secondary antibody at 1:400 dilution was used to visualise protein localisation together with a DAPI (Sigma-Aldrich) nuclear counterstain used at 0.1 μg/ml, incubated on sections for 30 min at room temperature. Sections were rinsed and mounted in vectashield-mounting media before visualisation and image capture using a Zeiss Confocal LSM 710 (Zeiss, Oberkochen, Germany).

Knockdown and overexpression of c-kit
Kit siRNA (L-042174-00) and non-targeting (D-001810-10-05) were purchased from Dharmacon RNA Technologies and used at 100 nM to transfect HC11 cells as described above. Full-length mouse *c-kit* complementary DNA was PCR amplified from mice complementary DNA using the following primers *c-kit* FOR: 5′-AGCCACCGCGATGAGAGGC-3′, REV: TGCTCAGGC ATCTTCGTGCAC using Phusion polymerase (Finnzymes Oy, Espoo, Finland) and cloned into pGEM-T easy (Promega, Madison, WI, USA), and then subcloned into pBABE puromycin (Morgenstern and Land, 1990), which was used to transfect BOSC-23 packaging cells to produce ecotropic retroviruses. Filtered viral supernatant plus 4 μg/ml polybrene was used to infect recipient, the murine HC11 cell line. Stably expressing cells were selected for using 1.5 μg/ml of puromycin (Sigma-Aldrich) for 10 days, following which mRNA overexpression was verified using quantitative real-time PCR. HC11 dome assays were performed as described above.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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