ABSTRACT
The HLH transcriptional regulator Id4 exerts important roles in different organs, including the neural compartment, where Id4 loss usually results in early lethality. To explore the role of this basally restricted transcription factor in the mammary gland, we generated a cre-inducible mouse model. MMTV- or K14-cre-mediated deletion of Id4 led to a delay in ductal morphogenesis, consistent with previous findings using a germ-line knockout mouse model. A striking increase in the expression of ERα (Esr1), PR and FoxA1 was observed in both the basal and luminal cellular subsets of Id4-deficient mammary glands. Together with chromatin immunoprecipitation of Id4 on the Esr1 and Foxa1 promoter regions, these data imply that Id4 is a negative regulator of the ERα signaling axis. Unexpectedly, examination of the ovaries of targeted mice revealed significantly increased numbers of secondary and antral follicles, and reduced Id4 expression in the granulosa cells. Moreover, expression of the cascade of enzymes that are crucial for estrogen biosynthesis in the ovary was decreased in Id4-deficient females and uterine weights were considerably lower, indicating impaired estrogen production. Thus, compromised ovarian function and decreased circulating estrogen likely contribute to the mammary ductal defects evident in Id4-deficient mice. Collectively, these data identify Id4 as a novel regulator of estrogen signaling, where Id4 restrains ERα expression in the basal and luminal cellular compartments of the mammary gland and regulates estrogen biosynthesis in the ovary.

KEY WORDS: Mammary gland, ID proteins, Estrogen, Ovary, Mouse

INTRODUCTION
The Id family of helix-loop-helix proteins comprises four members (Id1-Id4) that have emerged as important regulators of the balance between proliferation and differentiation in a number of different developmental systems (Lasorella et al., 2014; Sikder et al., 2003). Id proteins lack a DNA-binding motif and appear to function by sequestering basic-HLH factors from dimerizing with their partner proteins and thus preventing them from binding DNA to regulate transcription. Id4 is of particular interest because it has been shown to be important for development of the neural system, mammary gland and ovary. Id4 protein could also be demonstrated in this subset identified as luminal progenitors (CD29loCD24+CD49b+) (Fig. 1C), consistent with previous gene profiling studies (Lim et al., 2010). Id4 expression in the basal and luminal cellular compartments of the mammary gland and prostate using a germline knockout model. Only 20% of mice survive on an Id4-null background, following a high proportion of embryonic and post-partum lethality resulting from severe brain and neuronal abnormalities (Bedford et al., 2005; Yun et al., 2004). In the male reproductive system, Id4 loss results in significantly reduced seminal vesicle size, prostatic intraepithelial neoplasia (PIN) lesions and sterility (Sharma et al., 2013), while a recent study in the mammary gland has shown that Id4 governs ductal elongation and branching via the Id4 target p38MAPK (Dong et al., 2011). In breast epithelial cells, Id4 negatively controls expression of the key tumor suppressor Brca1 (Beger et al., 2001) and also suppresses miR335, a positive regulator of Brca1 (Heyn et al., 2011). In the context of breast cancer, overexpression of Id4 tightly correlates with the ‘triple-negative’ subtype (de Candia et al., 2006; Wen et al., 2012) and inversely correlates with expression of BRCA1 and estrogen receptor α (ERS; Esr1) (Molyneux et al., 2010; Roldán et al., 2006). There is mounting evidence that transcriptional regulators are essential for control of the mammary epithelial differentiation hierarchy (Visvader, 2009). As Id4 had been previously identified as a marker of the mammary stem (MaSC)/basal cell population (Dong et al., 2011; Lim et al., 2010), we generated a floxed Id4 mouse model to permit further analysis of the role of Id4 in mammopoiesis, in the absence of the early lethality that accompanies germline deletion of this gene. Here, we report a dual role for Id4 in regulation of the estrogen-signaling axis in both the developing mammary gland and ovary.

RESULTS AND DISCUSSION
Conditional deletion of Id4 results in a delay in mammary gland morphogenesis
To generate a conditionally targeted Id4 mouse model, the entire open reading frame of Id4 located in exons 1 and 2 was flanked with LoxP sites. A GFP reporter cassette enabled tracking of specific cell populations and proved to be a sensitive read-out of Id4 promoter activity (Fig. 1A,B; supplementary material Fig. S1A). FACS (fluorescence-activated cell sorting) analysis of GFP at different developmental time-points showed that endogenous Id4 expression was highest in the MaSC/basal population (CD29+CD24-) (Shackleton et al., 2006) at puberty (supplementary material Fig. S1B), consistent with previous gene profiling studies (Lim et al., 2010). Id4 may be expressed in multiple basal cell types, as this subset contains stem cells, progenitors and myoepithelial cells. Unexpectedly, a small population of luminal cells was found to express Id4-GFP (Fig. 1B). Using CD49b for further fractionation, these GFP+ cells were identified as luminal progenitors (CD29+CD24-CD49b+) (Fig. 1C), and Id4 protein could also be demonstrated in this subset (supplementary material Fig. S1C). Immunostaining confirmed high Id4 expression in the outer myoepithelial layer of both the mammary ducts and terminal end buds (TEBs) at early puberty (supplementary material Fig. S1D,E).

To assess the in vivo effects of deleting Id4 in mammary epithelial cells, we used two cre models, MMTV-cre or Keratin14-cre, both of
which resulted in efficient recombination at the Id4 locus (Fig. 1D,E; supplementary material Fig. S1F). The ductal elongation defects in MMTV-cre/Id4f/f and K14-cre/Id4f/f mammary glands recapitulated those previously described for germline knockout mice (Fig. 1F; supplementary material Fig. S1G) (Dong et al., 2011), with an intermediate phenotype observed for heterozygous mice. Although ductal elongation had been largely restored in MMTV-cre/Id4f/f glands by 8 weeks of age, complex branching of the ducts was still lacking (supplementary material Fig. S1H). To examine the repopulating capacity of Id4-deficient MaSCs isolated from mice at the onset of puberty when Id4 is prominently expressed in the TEBs, we transplanted the MaSC/basal population from early pubertal females. No difference in the frequency of repopulating units (Table 1) was evident for Id4f/+ versus MMTV-cre/Id4f/f mice, indicating that Id4 was not required for MaSC function at this stage.

Deregulation of ERα in Id4-deficient mammary glands

The ductal architecture of Id4-deficient mammary ducts was unperturbed based on a range of lineage-specific markers, including the luminal markers keratin 8 (K8) and ERα (Fig. 2A). Surprisingly, ERα+ cells were more abundant in ductal cells of MMTV-cre/Id4f/+ glands, relative to those of littermate controls (Fig. 2A,B). Immunostaining of freshly sorted, cytospun cells revealed that the proportion of ERα+ cells was increased in the luminal progenitor subset (CD29loCD24+CD49b+) from Id4-deficient mammary glands. Moreover, expression was also detected in the MaSC/basal population, which normally lacks expression of ER and PR (Fig. 2C,D; supplementary material Fig. S2A) (Asselin-Labat et al., 2006). The expression of other proteins in the ERα pathway (Welboren et al., 2009), including FoxA1, the pioneering factor for ERα (Hurtado et al., 2011), and the progesterone receptor (PR) (Tanos et al., 2012) were next evaluated by immunostaining. Parallel to findings for ERα, FoxA1 mRNA and protein were dramatically upregulated in Id4-deficient MaSC/basal cells compared with those from control mice (Fig. 2E,F; supplementary material Fig. S2B). Moreover, the number of PR+ ductal cells nearly doubled in MMTV-cre/Id4f/f epithelium (supplementary material Fig. S2C). FACS analysis of Sca1 expression, an antigen linked to ER and PR expression (Shehata et al., 2012), also showed a significant increase in Id4-deficient luminal progenitor cells (supplementary material Fig. S2D). Thus, Id4 appears to have an important role in restraining expression of ERα and its associated network of transcription factors in basal and luminal cells.

Table 1. Limiting dilution analysis of the mammary repopulating frequency of the CD29hiCD24+ subset from 3-week-old Id4f/+ and Id4MMTVΔ/Δ mice

<table>
<thead>
<tr>
<th>Number of cells injected per fat pad</th>
<th>Id4f/+</th>
<th>Id4MMTVΔ/Δ</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>14/20</td>
<td>14/27</td>
</tr>
<tr>
<td>200</td>
<td>17/20</td>
<td>19/22</td>
</tr>
<tr>
<td>400</td>
<td>19/20</td>
<td>10/11</td>
</tr>
<tr>
<td>Repopulating frequency</td>
<td>1/104</td>
<td>1/126</td>
</tr>
<tr>
<td>95% confidence interval</td>
<td>73.1-148</td>
<td>90-177</td>
</tr>
<tr>
<td>P value</td>
<td>0.432</td>
<td></td>
</tr>
</tbody>
</table>

Id4-deficient ovaries express reduced levels of estrogen biosynthesis enzymes

Although ERα was aberrantly expressed in the basal and luminal epithelial subsets, no mammary hyperplasia was apparent in aged Id4-deficient mice. Mouse models in which ERα is overexpressed generally develop hyperplastic glands and adenocarcinomas with a short latency (Frech et al., 2005; Tilli et al., 2003). We therefore
hypothesized that estrogen production in MMTV-cre/Id4f/f mice might be compromised in Id4-deficient mice to account for the ductal elongation defects. Analysis of ovaries from these mice at estrus revealed irregular-shaped nodular ovaries that contained many large follicles in a loosely connected medulla (Fig. 3A). Pertinently, gene expression studies have identified Id4 expression in the estrogen-producing granulosa cells of the ovary (Hogg et al., 2010; Johnson et al., 2008), and expression of MMTV-cre has been reported in the oocyte and reproductive tract (Wagner et al., 2001). Indeed, immunostaining confirmed that Id4 was expressed within the granulosa cells of secondary and antral follicles of ovaries from control mice but was absent in MMTV-cre/Id4f/f ovaries (Fig. 3B; supplementary material Fig. S3A).

The number of secondary and antral follicles as well as atretic (degenerating) follicles was increased in the ovaries of MMTV-cre/Id4f/f mice, although the total number of follicles per ovary was comparable to that in control mice (Fig. 3C; supplementary material Fig. S3B). Increased responsiveness of granulosa cells to the pituitary hormone follicle-stimulating hormone (FSH) could be inferred from the increased numbers of antral follicles. The elevated FSH-receptor transcript levels in Id4-deficient ovaries (Fig. 3D) are consistent with the increase in maturing follicle numbers and may mediate enhanced responsiveness to FSH.

Despite an overall increase in the number of growing and maturing follicles in the ovaries from Id4-deficient mice, we found that expression of key estrogen biosynthesis enzymes Cyp11a1 (cholesterol side chain cleavage enzyme), Star (steroidogenic acute regulatory protein), Cyp17a1 (17,20 lyase), HSD3β (3-β-hydroxysteroid dehydrogenase; Hsd3b) and Cyp19a1 (aromatase) were all significantly lower in Id4-deficient ovaries relative to controls (Fig. 3E). Conversely, expression of the key negative transcriptional regulator of the majority of these enzymes, Foxl2 (Escudero et al., 2010), was increased (Fig. 3F). In order to verify a reduction in estrogen production, we measured the weight of uteri. The uterus is extremely sensitive to estrogen levels and uterine weight is commonly used as measure of estrogen response (Liew et al., 2010). Notably, the wet uteri of Id4-deficient mice were significantly reduced compared with control mice (Fig. 3G), indicating diminished levels of circulating estrogen and an important role for Id4 in ovarian development.

To investigate whether the delayed ductal elongation in Id4-deficient mammary glands was a consequence of lower levels of systemic estrogen, we performed an estrogen-replacement experiment (supplementary material Fig. S3C). Using a regimen similar to that previously described (Gresack and Frick, 2006), we increased the amount of circulating estrogen in female mice during pubertal development and verified the increase via uterine wet weight measurements (supplementary material Fig. S3D). Importantly, ductal morphogenesis was rescued in MMTV-cre/Id4f/f females injected with estrogen, implying that these defects are not intrinsic to the mammary epithelium and likely result from low circulating estrogen levels (supplementary material Fig. S3E). The increase in ERα-positive luminal cells was still apparent in Id4-deficient mammary glands with higher circulating estrogen levels (supplementary material Fig. S3F). The wet uterus of Id4-deficient mammary glands was a consequence of lower levels of circulating estrogen

Reduced serum estrogen may account for the differences observed in the mammary repopulating frequencies for 12- to 16-week-old Id4f/f (Dong et al., 2011) versus young MMTV-cre/Id4f/f mice (Table 1). Stem cells isolated from older mice have likely been...
exposed to a low-estrogen environment for several weeks prior to transplantation, whereas this will not apply to young pubertal mice. Indeed, MaSCs from ovariectomized females show significantly reduced activity (Asselin-Labat et al., 2010).

**Id4 negatively regulates ERα protein expression**

To determine whether Id4 directly targets the *Esr1* gene in mammary epithelial cells, we performed chromatin immunoprecipitation (ChIP) analysis of endogenous Id4 protein in both primary sorted luminal cells and the CommaDβgeo cell line. Specific binding of Id4 occurred at a region located 5.9 kb upstream of the ERα promoter (Fig. 4A; supplementary material Fig. S3F). We presume that Id4 binds the ERα upstream regulatory region as part of a larger protein complex, as Id4 itself cannot bind to DNA. Furthermore, an Id4 site was identified 8.3 kb upstream of the *Foxa1* transcription start site, and perhaps forms part of a similar protein complex (Fig. 4B; supplementary material Fig. S3G). To further explore a transcriptional link between Id4 and ERα, we overexpressed Id4 in CommaDβgeo cells. Western blot analysis showed a consistent decrease in ERα protein expression in cells overexpressing the MSCV-Id4 construct compared with control cells (Fig. 4C), thus invoking a direct link between Id4 and ERα expression. In the context of tumorigenesis, although little difference in tumor latency was observed between Id4<sup>f/+</sup>/Wnt1 and K14-cre/Id4<sup>f/f</sup>/Wnt1 mice (data not shown), Id4<sup>-</sup>-deficient tumors expressed high levels of ERα and FoxA1 (Fig. 4D). As Wnt1 tumors express high levels of Id4 and have a signature reminiscent of the MaSC/basal population (ERα and FoxA1 negative) (Lim et al., 2010), these data underscore the importance of Id4 in regulation of the ER pathway.

In summary, our findings have revealed an unsuspected role for Id4 in negatively regulating ERα expression in basal and luminal cells of the mammary gland and in controlling the expression of key estrogen biosynthesis enzymes in the ovary. The independent role of Id4 in follicle development in the ovary likely results in reduced estrogen levels in Id4<sup>-</sup>-deficient mice with consequent effects on mammary gland development.

**MATERIALS AND METHODS**

**Mice**

Floxed-Id4 mice were generated by Ozgene; MMTV-cre (line A) mice were a gift from K.-U. Wagner (University of Nebraska Medical Center, USA);
K14-cre mice were a gift from J. Jonkers (Netherlands Cancer Institute, The Netherlands). MMTV-cre and K14-cre mice were maintained on a FVB/N background and floxed-Id4 mice were analyzed on a mixed FVB/N C57/B16 background. All animal experiments conform to regulatory standards and were approved by the Walter and Eliza Hall Institute (WEHI) Animal Ethics Committee. Vaginal smears were taken with saline and counterstained with hematoxylin and eosin. Uterus wet weight measurements taken from single horns (proestrus or estrus), with adipose and ovary removed.

17β-Estradiol (Sigma-Aldrich) in ethanol was diluted in sunflower seed oil (Sigma-Aldrich) to 4 μl/g for subcutaneous injection into mice at 0.2 mg/kg body weight. Mouse tail DNA was genotyped using primers (shown in supplementary material Table S2) with apolipoprotein E and ovary removed.

Histology and immunohistochemistry
Inguinal mammary glands fixed in Carnoy’s solution overnight were stained in Carmin Alum. Whole-mounts were quantified using ImageJ software (Softonic). Mammary glands or ovaries were fixed in 4% paraformaldehyde and stored in 70% ethanol prior to histology facilities at WEHI.

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study. F.V. performed fat pad transplantations. N.Y.F. performed validation experiments on the targeted model. L.H. performed ovary dissection. S.H.L., K.J.H. and C.L.S. discussed and performed follicle analysis and counts. S.A.B. and J.E.V. wrote the manuscript.

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