Oestrogen receptor-alpha regulates non-canonical Hedgehog-signalling in the mammary gland

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A B S T R A C T

Mesenchymal dysplasia (mes) mice harbour a truncation in the C-terminal region of the Hh-ligand receptor, Patched-1 (mPtch1). While the mes variant of mPtch1 binds to Hh-ligands with an affinity similar to that of wild type mPtch1 and appears to normally regulate canonical Hh-signalling via smo, the mes mutation causes, among other non-lethal defects, a block to mammary ductal elongation at puberty. We demonstrated previously Hh-signalling induces the activation of Erk1/2 and c-src independently of its control of smo activity. Furthermore, mammary epithelial cell–directed expression of an activated allele of c-src rescued the block to ductal elongation in mes mice, albeit with delayed kinetics. Given that this rescue was accompanied by an induction in estrogen receptor-alpha (ERα) expression and that complex regulatory interactions between ERα and c-src are required for normal mammary gland development, it was hypothesized that expression of ERα would also overcome the block to mammary ductal elongation at puberty in the mes mouse. We demonstrate here that conditional expression of ERα in luminal mammary epithelial cells on the mes background facilitates ductal morphogenesis with kinetics similar to that of the MMTV-c-srcK44E mice. We demonstrate further that Erk1/2 is activated in primary mammary epithelial cells by Shh-ligand and that this activation is blocked by the inhibitor of c-src, PP2, partially blocked by the ERα inhibitor, ICI 182780 but is not blocked by the smo-inhibitor, SANT-1. These data reveal an apparent Hh-signalling cascade operating through c-src and ERα that is required for mammary gland morphogenesis at puberty.

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Introduction

Estrogens are a class of hormones that play crucial roles in diverse aspects of human biology, including sexual development and reproduction. Dysregulation of estrogen signalling has been implicated in a variety of diseases, including breast and uterine cancers, osteoporosis and cardiovascular disease. Estrogen receptor-alpha (ERα) is one of the principal protein receptors responsible for mediating the actions of estrogen (Heldring et al., 2007). As a member of the large superfamily of nuclear hormone receptors, ERα acts as a ligand-activated transcription factor (Heldring et al., 2007; Mangelsdorf et al., 1995). Upon binding of its ligand, 17β-estradiol (E2), ERα modulates transcription by either binding directly to estrogen response elements (ERE) in the promoter of E2 regulated genes, or indirectly through protein–protein interactions with other transcription factors (Heldring et al., 2007; Klinge, 2000).

ERα regulat[es target gene transcription through two independent activation functions (AF), AF-1 and AF-2 (Arao et al., 2011; Arnal et al., 2013; Lannigan, 2003; McGlynn et al., 2013). Transcriptional activation by ERα can be promoted through functional cooperation between the two AFs or through either AF independently (Arnal et al., 2013). AF-2 contains the ligand binding domain (LBD), and its activity depends on E2 binding (Arao et al., 2011; McGlynn et al., 2013). Ligand-independent activation is facilitated by the AF-1 domain. This domain harbours several sequences that facilitate phosphorylation by factors mediating a number of signal transduction pathways. For example, MAPK and Cdk7 activates ERα by directly phosphorylating Ser118 within the AF-1 domain (Bunone et al., 1996; Chen et al., 2002). More recently, it was determined that E2 elicits changes in cellular processes through ERα outside of the nucleus via activation of protein kinases, phosphatases and secondary messengers (Haynes et al., 2003; Koustev et al., 2001). One of these rapid “non-genomic” signal transduction mechanisms includes the c-src protein tyrosine kinase (Castoria et al., 2012; Li et al., 2007). ERα transiently activates c-src in response to E2 binding by increasing phosphorylation of Tyr416, resulting in downstream activation of...
Morphogenesis not only requires E2-activated ERα derived from c-src-null mice fail to respond to exogenous estrogen (Lubahn et al., 1993; Pedram et al., 2009). Interestingly, animals lacking detectable c-src also exhibit defects in mammary gland morphogenesis and primary mammary epithelial cells (MECs) derived from c-src-null mice fail to respond to exogenous estrogen stimulation (Kim et al., 2005). Thus, proper mammary gland morphogenesis not only requires E2-activated ERα, but also depends on ERα interaction with other signalling cascades.

We and others demonstrated previously that Hedgehog (Hh)-signalling also plays a role in mammary gland development (Chang et al., 2012; Moraes et al., 2009). Animals homozygous for the mesenchymal dysplasia (mes) (Makino et al., 2001; Sweet et al., 1996) allele of the Hh-ligand receptor, Patched-1 (Ptch1), displayed a blocked mammary gland phenotype resembling that of the ERα knock-out mice. The lack of ductal outgrowth in mes mice was associated with reduced expression of ERα and progesterone receptor (PR) in epithelial cells (Chang et al., 2012; Moraes et al., 2009). The mes allele encodes a deletion in the second-last exon of Ptch1, resulting in a truncated protein that replaces the last 220 a.a. with a random 68 a.a. polypeptide. We showed that this region of Ptch1 binds to factors containing SH3- and WW-domains, that the SH3-domain of c-src binds to the C-terminus of mPtch1 (Chang et al., 2010) and that transiently expressed mPtch1 binds to endogenous c-src in the absence of added Shh-ligand (Harvey et al., 2014). Using a genetic approach, we showed further that forced expression of an activated c-src (c-src<sup>act</sup>) transgene in luminal mammary epithelial cells rescued the blocked mammary morphogenesis arising in mes mice (Chang et al., 2012). This rescue was accompanied by a strong increase in ERα expression.

Interactions between the ERα activity and Hh-signalling has also been demonstrated. In ERα-positive gastric cancer cells, E2 induced Shh expression and promoted cellular proliferation independent of smo-activity (Kameda et al., 2010). A similar result was reported in the ERα-positive breast cancer cell line, MCF-7. Here, E2 induced expression of Shh and Gli1. This activation is inhibited in cells treated with the anti-estrogen, ICI 182780 (Koga et al., 2008). Inhibiting smo-activity with the small molecule inhibitor, cyclopamine, significantly suppressed proliferation of both ERα-positive and ERα-negative breast cancer cell lines, MCF-7 and MDA-MB-231, respectively (Che et al., 2013). Cyclopamine also significantly decreased ERα expression in MCF-7 cells. Since c-src binds to mPtch1 (Chang et al., 2010; Harvey et al., 2014) and also activates ERα (Castoria et al., 2012; Sun et al., 2012), we sought to determine whether ERα-activity acts genetically downstream of Ptch1. We demonstrate here that conditional expression of ERα overcomes the block to ductal elongation in mes mice with kinetics similar to those for the rescue of the mes phenotype by the MMTV-c-src<sup>act</sup> allele. Furthermore, we define a novel pathway stimulated by Shh that activates Erk1/2 and requires the activity of either ERα or c-src but not smo.

Materials and methods

**Cell culture.** HEK 293 cells (a gift of S. Girardin) were cultured in DMEM with 10% FBS and 1% penicillin-streptomycin. Shh Light II fibroblasts (ATCC) were cultured in DMEM supplemented with 400 mg/ml G418 (Gibco) and 0.14 μg/ml Zeocin (Invitrogen).

**Mice.** Wild type C57Bl/6 mice (Charles River) or C57Bl/6N mice heterozygous for the mesenchymal dysplasia (mes) allele of Ptc1 (JacksonLabs) were crossed with CER Mice, which harbour a flag-tagged ERα transgene under the control of the tet<sup>fl</sup> and the “Tet-On” transgene under the control of the MMTV promoter (Díaz-Cruz et al., 2011; Frech et al., 2008, 2005; Hruska et al., 2002; Miermont et al., 2010) (P. A. Furth, Georgetown University). Compound mice were then backcrossed onto C57Bl/6 (CharlesRiver) for >4 generations. Transgene expression was induced by constant administration (changed twice per week) of 2 mg/ml doxycycline in sterile-filtered drinking water containing 5% sucrose.

For genotyping, tail DNA was extracted and 2 μl DNA was amplified in a 25 μl polymerase chain reaction using Taq DNA Polymerase (Thermo Scientific). Forward (F) and reverse (R) primer sequences were as follows:

**Meso.** F: 5′-TCCAAGGTGCTCCGCGTTG-3′ and R 5′-GTTGCCCTCCA-CAATCAGCTTCTG-3′ (Chang et al., 2012); FLAG-ERα: F 5′-CGAGCTCCTC-GTACCCGCTGC-3′ and R 5′-GAACAGCTGGCTTGTTG-3′ (Miermont et al., 2010); MMTV-tetRα: F 5′-ATCCGCCACCTTGATGA CTCGG-3′ and R 5′-GGCTATACACCAACTGCGAC-3′ (Miermont et al., 2010). Reaction conditions for MMTV-tetRα and mes were 60 s each for denaturation, annealing and extension for 32 cycles, while for FLAG-ERα they were 60 s denaturation, 90 s annealing, and 120 s extension for 35 cycles. The annealing temperatures for mes, FLAG-ERα and MMTV-tetRα were 53 °C, 57 °C and 56 °C, respectively.

**Whole mount analysis.** Whole mount analysis of mammary glands was performed as previously described (Chang et al., 2012; Rasmussen et al., 2000). Briefly, mammary glands were fixed in Carnoy’s fixative (10% acetic acid, 30% chloroform, 60% ethanol) overnight at 4 °C, and washed in 70%, 50% and 25% ethanol for 15 min each and then stained in Carnine alum overnight at room temperature. The glands were washed in 70%, 95% and 100% ethanol for 15 min each and then cleared in xylene for two changes for 30 min each followed by mounting with Permount.

**Immunofluorescence.** Paraffin-embedded tissue sections were deparaffinized in xylene and rehydrated gradually through 100%, 95%, 80%, 70% ethanol washed and water. Antigen retrieval was performed by boiling sections for 15 min in 10 mM sodium citrate, pH 6.0, in a pressure cooker pre-heated in a microwave oven for 20 min before immersing slides. After boiling, sections were permeabilized with 0.2% TritonX-100 for 10 min. After washing, sections were incubated with primary antibodies for 1 h at room temperature in 3% BSA in PBS. Antibodies and dilutions are as follows: 1:100 anti-ERα (Santa Cruz; sc-542), 1:100 anti-BrdU (abcam: ab6326), 1:20 anti-Flag (abm;G191) and undiluted anti-AAX4 containing supernatant (produced in our lab). Sections were then washed and labelled with fluorescently-labelled secondary antibody added to samples for 1 h. Sections were imaged using a Nikon Eclipse fluorescence microscope equipped with a QImaging Fast1394 digital camera and compiled using QCapture Pro software (QImaging).

**Preparation of Shh-conditioned media.** Shh-conditioned media was prepared as previously described (Capuro et al., 2012). Shh- and pDNA-conditioned media were prepared by transfecting 40% confluent 10 cm plates of HEK293 cells in 10% FBS with 10 μg of pDNA3.1-N-Shh (gift of J. Filmus, Sunnybrook Health Sciences Centre) or pDNA3 using 2 mg/ml PEI at a 2:1 ratio. Cells were grown for 24 h before the media was switched to 5% FBS for 48 h. The media was collected, centrifuged at 2500 rpm for 5 min at 4 °C and harvested. The supernatant was then sterile-filtered using a 0.22 μm syringe filter. Prior to use, conditioned media was diluted
in serum-free DMEM to a final serum concentration of 0.5%. Activity of conditioned media was measured in a luciferase assay in Shh Light II fibroblasts (Chang et al., 2010; Sasaki et al., 1997).

**Primary cell culture.** Primary mammary epithelial and mesenchymal cells were isolated as described previously (Chang et al., 2012; Niranjan et al., 1995). Briefly, thoracic and inguinal mammary glands were dissected from euthanized mice and minced with a razor blade into small fragments. The minced mammary glands were incubated in a solution of 3 mg/ml collagenase A and trypsin (Sigma-Aldrich) in DMEM/F12 for 2 h at 37°C. Mechanical dissociation was then performed by slowly pipetting with a 5 ml pipette, adding FBS to a final concentration of 2%. Epithelial (pelleted) and mesenchymal (supernatant) layers were separated by centrifuging at 200 rpm for 2 min. The mesenchymal layer was collected and washed with DMEM/F12 and plated in DMEM/F12 containing 10% FBS supplemented with 10 μg/ml insulin, 10 μg/ml transferrin and 20 ng/ml EGF. For organoid culture, the epithelial pellet was resuspended in 3 mg/ml collagenase A in DMEM/F12 and incubated for 30 min at 37°C. The cells were pelleted by centrifugation, washed and plated in primary culture media mentioned above. The organoid containing media was removed and re-plated once the remaining mesenchymal cells attached to the plate (~2 h).

For Erk1/2 activation assays, primary mouse mammary epithelial cells and mesenchymal cells were serum starved in 0.2% FBS containing culturing media for 24 and 48 h, respectively. Cells were then stimulated with pcDNA3-conditioned media, Shh-conditioned media, or Shh-conditioned media with either 10 nM ICI 182 780, 20 nM SANT-1 (Toronto Research Chemicals), or 10 nM conditioned media, or Shh-conditioned media with either 10 nM ICI 182 780, 20 nM SANT-1 (Toronto Research Chemicals), or 10 nM PP2 for 24 h. Cells were lysed in 1% NP40 lysis buffer and levels of phospho-Erk1/2 and total Erk1/2 determined by western blot.

**Western blotting.** Cell lysates were prepared following addition of 1% NP40 lysis buffer containing protease and phosphatase inhibitors (0.57 mM PMSF, 10 μM leupeptin, 0.3 μM aprotinin, 10 mM NaF, 1 mM sodium orthovanadate). For western blots, 4 x SDS-loading buffer (50 mM Tris pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol) was added to 50 μg of lysate and boiled for 5 min. Samples were resolved by 10% SDS-PAGE and blotted onto nitrocellulose membrane. Blots were probed with primary antibody overnight at 4°C. Antibodies and dilutions used are as follows: 1:1000 mouse α-p-ERK (Cell Signaling) and 1:1000 rabbit α-ERK (Cell Signaling). Blots to be re-probed were stripped in stripping buffer (2% SDS, 62.5 mM Tris pH 6.8, 100 mM β-mercaptoethanol) for 30 min at 72°C.

**Quantitative RT-PCR.** For quantitative RT-PCR (qPCR), total RNA was isolated from primary mouse mammary mesenchymal and epithelial cells using Trizol (Invitrogen). DNase treatment (Fermentas) was performed on 400 ng of RNA from each sample. Reverse transcription was performed using SuperScript II Reverse Transcriptase (Invitrogen) and random hexamer primers (Fermentas). Resulting cDNA was diluted 1:25 and qPCR was then performed using an iQ SYBR Green Supermix (Bio-Rad) in 10 μl reactions. All reactions consisted of 40 cycles with 30 s denaturation at 72°C, 30 s annealing and 30 s extension at 60°C. Primer specificity was confirmed by a melt curve analysis. Results were quantified using the ΔΔCt method with Arbp as a reference gene. Primers adapted from previously published sequences (Webster et al., 1995; Zhang et al., 2009) were used as follows:

- mPtch1 forward: 5′-GGTGTTTCATCAAAGTTCG-3′
- mPtch1 reverse: 5′-GGCATAGGCAAGCATCAGTA-3′
- mGli1 forward: 5′-CCCATAGGGTCTCGGGGTCTCAAAC-3′
- mGli1 reverse: 5′-GGGACCTGCGGCTGACTGTGTAA-3′
- mArbp forward: 5′-GAAAAATCTCCAGAGGCACCATTG-3′
- mArbp reverse: 5′-TCCCACCTTGCTCCAGTTTAT-3′

**Results.**

Mice homozygous for the mes allele exhibit a block to ductal elongation in the mammary gland during puberty. This defect is overcome, albeit with distinct kinetics relative to wild type animals, by constitutive expression of activated c-src under the control of the MMTV-promoter. Given the increased expression of

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*Fig. 1.** Forced expression of Erα rescues the mammary gland phenotype in mes mice. Mammary gland #4 was isolated from twenty week-old mice and whole mounts prepared. (A) and (A’) mes; (B) and (B’) mes/CERM. After twenty weeks, mammary ductal morphogenesis in mes mice is blocked. This phenotype is reversed in mes mice expressing Erα directed to mammary epithelium, albeit with delayed kinetics. Magnification of ducts reveal a non-proliferative rudimental ductal structure in mes mammary glands (A’). Terminal end buds are present and ductal structures have not reached the limits of the fat pads in mes/CERM mice (B’).*
ERα in mes/MMTV-c-srcAct mice and the role of c-src in activating ERα, we hypothesized that forced expression of ERα would also rescue the mes phenotype. CERM mice (Frech et al., 2005; Tilli et al., 2003), a compound transgenic mouse that harbours a tetO-ERαFlp allele under the control of MMTV-“Tet-On”, were bred to mes mice and backcrossed onto a C57Bl/6 background. ERα expression in compound mes/CERM mice was then induced by long-term administration of the doxycycline beginning at 3 weeks of age. As Fig. 1A and A’ illustrate, at 20 weeks of age, mammary gland #4 in the adult mes females had not developed, remaining in the state that developed during embryogenesis. However, as Fig. 1B shows, ductal elongation was apparent in compound mice homozygous for the mes allele and expressing MMTV-directed ERα (mes/CERM). At this stage, terminal end buds were also apparent (Fig. 1B’) indicative of these ducts elongating into the fat pad similar to that observed for wild type mice at puberty.

Similar to the observation we reported previously for mes/MMTV-c-srcAct mice (Chang et al., 2012), the rescue by CERM mice of the mes phenotype exhibited distinct kinetics of ductal elongation relative to the development of wild type mammary glands (Fig. 2). By ten weeks of age, only rudimentary ductal structures resembling those of their mes littermates were seen for doxycycline-fed mes/CERM mice (Fig. 2A). However, between 15 and 20 weeks, the mammary ducts in doxycycline-fed mes/CERM mice had penetrated the fat pad to various degrees (Fig. 2B). Significantly, terminal end buds (TEBs) were present in these animals (see Fig. 1B’). Complete morphogenesis was observed in doxycycline-fed mes/CERM females between 24 to 30 weeks of age (Fig. 2C). For these mature animals, the ducts in these mammary glands reached the limits of the fat pad and TEBs were no longer present. As we reported previously (Chang et al., 2012), development of the mammary glands in mes littermates beyond the primitive ductal structures established during embryogenesis was not observed, even at thirty weeks (Fig. 2E) nor were ducts evident in mes/CERM animals not induced with doxycycline (Fig. 2F).

To determine if the mammary ducts of mes/CERM mice exhibited normal epithelial organization, sections were probed for ERα and the myoepithelial marker, p63 (Fig. 3). Nuclear ERα expression was observed in mammary epithelial cells from ducts of adult virgin wild type, mes, wild type/CERM and mes/CERM mice. Expression of ERα was restricted to the luminal epithelial cells, evident by the lack of overlap between signals for ERα with p63. As we demonstrated previously (Chang et al., 2010; Moraes et al., 2009), reduced ERα expression was observed in mes mice (Fig. 3E and H) compared to wild type littermates (Fig. 3A–D). While approximately 40% of epithelial cells were positive for ERα staining in wild type animals, ERα staining was only observed in 10–20% of epithelial cells in mes littermates. An approximate 2-fold increase in the percentage of cells demonstrating nuclear localized ERα expression was observed in mes mice expressing MMTV-ERα, relative to mes alone. However, expression levels and subcellular localization of ERα were indistinguishable between wild type (Fig. 3A–D), mes/CERM (Fig. 3M–P) and WT/CERM (Fig. 3I and L) littermates.

Immunofluorescence revealed that induction of the conditional ERα expression in both wild type and mes mice caused aberrant cell cycle progression in adult mammary glands (Fig. 4). Specifically, cells incorporating BrdU were essentially absent in the mammary glands of wild type adult animals (Fig. 4A and D).
However, following the 2 h pulse of BrdU, a fraction of cells in every duct in doxycycline-fed WT/CERM (Fig. 3E and H) and mes/CERM (Fig. 3M–P) mice stained positively, indicating the presence of cycling cells due to the induced expression of ERα. BrdU incorporation was not observed in WT/CERM mice not fed doxycycline (Fig. 4I and L). Cells expressing detectable levels of the flag-tagged ERα transgene were not associated with the cells that had incorporated BrdU (Supplementary Fig. 1), although all BrdU-incorporating cells stained with an anti-ERα antibody (Fig. 4C and D), suggesting that transgene expression generated an overall proliferative state in the mammary gland rather than driving specific cells through the cell cycle.

To further investigate the overall proliferative state of the mammary gland, changes in the expression of the stromally-restricted, homeodomain protein, Alx4 (Hudson et al., 1998; Qu et al., 1998, 1997a, 1997b), were assessed. Alx4 is induced in stromal fibroblasts during puberty and pregnancy but is not expressed in the resting adult mammary gland (Hudson et al., 1998; Joshi et al., 2006). Furthermore, administration of E2 to prepubescent female mice induces Alx4 expression in these fibroblasts (Hudson et al., 1998; Joshi et al., 2006). Shh-ligand also stimulates the activation of both Erk1/2 (Chang et al., 2010) and c-src (Harvey et al., 2014) in a human mammary epithelial cell line, MCF10a, that lacks detectable expression of smo (Chang et al., 2012, 2010; Mukherjee et al., 2006; Zhang et al., 2009). As Fig. 6A illustrates, phosphorylation of Erk1/2 was induced in wild type, serum-starved primary mammary epithelial cells within 15 min of stimulation by N-Shh. Stimulation occurred in the presence of the smo-antagonist, SANT-1, which we showed...

Fig. 3. mes/CERM ducts demonstrate normal cellular organization and ERα expression. Immunofluorescence staining for ERα and p63 in adult virgin wild type ((A)–(D)), mes/mes ((E)–(H)), wild type/CERM ((I)–(L)) and mes/CERM ((M)–(P)) mice. No co-localization of ERα is observed with p63 ((D), (H), (L) and (P)). Reduced ERα expression is apparent in mes mice (F) compared to wild type (p < 0.02) (B). ERα expression is increased, however, in mes mice expressing MMTV-ERα (p < 0.021) (N), but expression levels do not differ between mes/CERM and wild type littermates. Scale bar: 25 μm. ERα-positive cell percentages were determined by cell counting; 10 fields per animal, n = 3 for all animal groups. Data was analyzed by one-way ANOVA followed by pairwise comparisons of means using Dunnnett’s multiple comparison’s test.
Fig. 4. Forced ERα expression induces cell cycle progression in adult mammary glands. Twenty four week-old wild type ((A)–(D)), induced wild type/CERM ((E)–(H)), uninduced wild type/CERM ((I)–(L)) and mes/CERM ((M)–(P)) littermates were injected with BrdU 2 h prior to mammary gland excision and incorporation was determined through immunofluorescence. BrdU incorporation was absent in the mammary glands of 24 week-old adult wild type mice (B). Cells positive for BrdU staining are observed in ducts from both induced wild type/CERM (F) and mes/CERM mice (N), but not in wild type/CERM that were not fed doxycycline (J). BrdU positive cells co-localize with cells expressing ERα ((H) and (P)). Scale bar: 25 μm.

Fig. 5. Conditional expression of ERα induces stromal expression of Alx4 in adult female mice. Mammary glands of wild type ((A)–(B)), wild type/c-src<sup>cre+</sup> ((C)–(D)), wild type/CERM ((E)–(F)), mes ((G)–(H)), mes/c-src<sup>cre+</sup> ((I)–(J)), mes/CERM (induced) ((K)–(L)) virgin 24 week-old adult female mice were stained for Alx4. Staining is absent in wild type mammary ducts. Expression of constitutive c-src<sup>cre+</sup> or conditional ERα in ductal epithelial cells induces stromal expression of Alx4. Scale bar: 25 μm.
previously inhibited canonical Hh-signalling in primary mammary mesenchymal cells (Harvey et al., 2014). Activation of Erk1/2 occurring through a smo-independent mechanism (Fig. 6E) is also consistent with our previous observations using the smo-deficient MCF10a breast cancer line (Chang et al., 2010) as well as in smo-deficient MEFs (unpublished observation). Thus, the dependence of c-src activity on N-Shh stimulation of Erk1/2 was determined in primary mammary epithelial cells treated with the c-src inhibitor PP2 (Hanke et al., 1996). As Fig. 6E shows further, a complete block of Erk1/2 activation occurs when c-src activity is inhibited.

The role of ERα for N-Shh activation of Erk1/2 was then determined by stimulating cells in the presence of the anti-estrogen, ICI 182 780 (Wakeling et al., 1991). As illustrated in Fig. 6C, ICI 182780 partially inhibited Erk1/2 activation by N-Shh ligand. Specifically, treatment of primary epithelial cells with N-Shh-conditioned media caused a 4-fold increase in Erk1/2 phosphorylation. This level of activation was reduced by 50% in the presence of ICI 182780. Thus, N-Shh activated Erk1/2 in the presence of the smo-inhibitor, SANT-1, but required the activities ERα and c-src.

While N-Shh stimulates non-canonical Hh-signalling cascades in both primary mammary epithelial and mesenchymal cells, smo-dependent canonical Hh-signalling in primary epithelial cells has not been reported. Thus, primary epithelial and mesenchymal cells from wild type mammary glands were stimulated with N-Shh peptide and changes in expression of both Hh-pathway targets, Gli1 and Ptch1 were determined. As Fig. 7A reveals, stimulation of primary mammary mesenchymal cells resulted in a greater than 25-fold increase in Gli1 expression. Likewise, Fig. 7B illustrates the 4-fold increase in Ptch1 expression in these same cells. We demonstrated previously that Gli1 and Ptch1 induction in primary mouse mesenchymal cells through N-Shh stimulation was dependent on the activity of smo, since induction is abolished in cells treated with N-Shh in the presence of SANT-1 or by using heat-killed N-Shh peptide (Harvey et al., 2014). However, despite the apparent response of these mesenchymal cells to N-Shh ligand, a very limited or no induction of Gli1 and Ptch1, respectively, were observed for primary epithelial cells. Specifically, a small but significant 3-fold increase in Gli1 levels was observed after 24 h while no induction of Ptch1 was observed. Thus, mammary epithelial cells are relatively refractory to stimulation through the canonical Hh-pathway but are able to respond through non-canonical pathway, determined by the activation of Erk1/2 by N-Shh.

Taken together, using both a genetic approach and experiments using primary mammary cells in culture, these data suggest that a
Further evidence of Hh-pathways operating independently of smo was revealed in the mesenchymal dysplasia (mes) mouse (Sweet et al., 1996). The 32-bp deletion in this animal in the sequence encoding the mPtch1 C-terminus causes a truncation in this region of mPtch1 and the addition of a small amount of an unrelated, random sequence (Makino et al., 2001). A number of non-lethal defects arise in this mouse, including a block to mammalian gland morphogenesis during puberty. As we have shown, this defect is not due to the lack of ovarian hormones but rather to the apparent unresponsiveness in cells of the mammalian gland to these factors (Chang et al., 2012; Moraes et al., 2009). Despite these defects, the mes variant of the mPtch1 protein binds Shh-ligand with an affinity similar to the wild type mPtch1 (Nieuwenhuis et al., 2007) and appears to facilitate signalling through the canonical Hh-signalling pathway similar to cells with the full length protein (Harvey et al., 2014). Thus, in the context of the canonical Hh-pathway, the basis for blocked morphogenesis in the mes mammalian gland is not apparent.

We hypothesized and showed previously, however, that the defect in the mes mammalian gland could be rescued, albeit with delayed kinetics, by expressing an activated version of c-src (Chang et al., 2012). Among changes in the mammalian glands of the mes/MMTV-c-srcAct mice was a significant increase in the levels of expression of Erk relative to mes mice alone and its accumulation in the cytoplasm of mammalian epithelial cells. These data suggested that Hh-signalling in the mammalian gland may require, at least in part, signalling through pathways that controlled the expression and activities of Erks and that forced expression of Erks in mes mice might also rescue the mes mammalian phenotype. As we demonstrated here, conditional expression of an Erk transgene under the control of the MMTV promoter also overcame the block to mammalian morphogenesis with kinetics similar to that of the MMTV-c-srcAct allele. Thus, these two distinct mouse models have revealed a novel Hh-signalling pathway, involving c-src and Erks, that plays an important role in postnatal mammalian gland morphogenesis.

We note that the phenotype of the mes mice we reported in this and our previous paper (Chang et al., 2012) differs from a previous report (Moraes et al., 2009). In the latter paper, the blocked development of the mammalian gland at puberty was not as robust as we have observed. The reason for the differences between these phenotypes is not apparent, particularly given that both labs backcrossed their animals onto C57Bl/6 backgrounds, albeit, using C57Bl/6 mice from colonies that first diverged in 1951 (Charles River (C57Bl/6N) versus Jackson Labs (C57Bl/6)). We have characterized almost 40 mes mice on the C57Bl/6N background and have found only 3 with partial ductal development of mammalian gland #4. Furthermore, for both the mes/MMTV-c-srcAct and mes/CERM animals, ductal growth was not apparent until after about 15 weeks, again supporting the stronger penetrance of mes mammalian phenotype in our colony. Similar to Moraes et al., however, we observed that mixing FVB onto the mes/C57Bl/6N background had no effect on the mes phenotype (unpublished observation).

Our data also showed that stimulation by N-Shh of primary mammalian epithelial cells activated Erk1/2 in both an Erk and c-src-dependent manner. Inhibition of activation of either factor, but not inhibition of smo, reduced or blocked the ability of N-Shh to stimulate Erk1/2. Thus, the non-canonical Hh-signalling pathway we identified previously appears to involve changes in the activities of Erks and c-src in mammalian epithelial cells. The precise mechanism remains undefined. It is clear, however, that the activities of both c-src and Erks are required for the N-Shh-dependent activation of Erk1/2. In the presence of inhibitors that blocked the activities of Erks or c-src, respectively, N-Shh-dependent Erk1/2 activation was attenuated or blocked completely while treatment with the small

**Discussion**

The Hedgehog-signalling pathway regulates morphogenesis in a large number of tissues. Detailed genetic and molecular studies have shown further that control of these processes by the Hedgehog ligands is typically mediated by control of the activities of the Patched-1 receptor that, in turn, indirectly controls the activity of smo. Interestingly, the primary sequence of mPtch1 predicts that, while potentially harbouring a sterol-sensing domain (Carstea et al., 1997; Loftus et al., 1997), signalling cascades involving the direct association of SH3 or WW-domains with Ptch1 may also be involved in Hh-signalling. Our previous studies showed that a number of factors harbouring these domains can associate with the cytoplasmic C-terminal region of mPtch1 (Chang et al., 2010). These include the SH3-domains of signalling factors such as c-src, Grb2 and PIK3R2 (p85α) as well as factors harbouring WW-domains such as the E3 ubiquitin ligases, WWP2 and Smurf2. Supporting this was the recent observation that Drosophila Smurf (dSmurf) complexes Drosophila Patched (dPtch) (Huang et al., 2013).

**Fig. 7.** Limited canonical Hh-signalling in primary mammalian epithelial cells. Primary mammalian epithelial and mesenchymal cells isolated from three month-old wild type mice were serum starved for 24 h and 48 h, respectively. Cells were then stimulated with heat-killed N-Shh peptide, active N-Shh peptide, or N-Shh peptide with 20 nM SANT-1 for 24 h. (A) qPCR reveals that Gli1 is induced 25-fold in mesenchymal by N-Shh. Only a small but significant 3-fold increase in Gli1 was seen in epithelial cells from the same animals, however. (B) N-Shh peptide induced Ptch1 expression 3.5-fold in mesenchymal cells. No induction of mPtch1 was observed for epithelial cells. Data were analyzed by one-way ANOVA followed by pairwise comparisons of means by Dunnett’s multiple comparisons test. Data are displayed as mean ± SE, *p < 0.05, n = 3.
molecule inhibitor of smo, SANT-1, had no effect. Thus, these data support the existence of a pathway stimulated by the Hh-ligands in mammary epithelial cells that operates through both c-src and ERα resulting in the activation of Erk1/2.

In both MMTV-c-srcAkt and in CLEM mice on the mes background, a significant increase in the levels of ERα relative to mes mice alone are observed in the mammary epithelial cells. The altered expression of ERα suggested that the level of its expression required for ductal morphogenesis at puberty in response to 17β-estradiol requires the normal activity of the mPtc1 C-terminus. This model does not preclude the possibility that the canonical Hh-pathway, signalling through smo, is also required. This possibility has not been strictly tested in the mouse mammary gland (i.e. conditional or tissue-specific knock out of smo in mammary epithelial and/or mesenchymal cells). However, mice harbouring conditional c-srcAkt or ERα alleles are sufficient to overcome the block to the pubescent mammary morphogenesis and is associated with increased levels of ERα relative to mes mice alone.

Our data also revealed the mPtc1-dependent activation of the mammary stromal cells in vivo. Specifically, Ax4-expression was probed in mammary glands of wild type mice expressing mammary epithelial-restricted ERα or c-srcAkt. In adult mice taken at 24 weeks, the stromally-restricted paired-like homeodomain transcription factor, Ax4, was induced in mammary fibroblasts whereas the wild type adults do not express Ax4 in these cells. We conclude that ERα or c-src expression in the luminal cells induced the expression of factors that stimulated the surrounding stromal cells. Interestingly, despite the rescue by ERα and c-src in the mes mice, induction of Ax4 expression was not observed in mes stromal cells. Thus, wild type mPtc1 appears to be required for stromal induction of Ax4 by adjacent mammary epithelial cells. The basis for the difference between mes and wild type mice has not been defined. We propose the possibility that ERα and c-src induce the expression of a Hh-ligand in mammary epithelial cells. This ligand then stimulates adjacent mesenchymal cells to express Ax4 but is dependent on the activities of wild type mPtc1 in these cells. This model can be tested in reciprocal transplantation experiments between mes and wild type mammary glands. Regardless, these data offer further evidence for the role of components of the Hh-pathway in mediating signalling interactions between stromal and epithelial compartments in the developing mammary gland.

While the focus of this study was on Hh-signalling that operates, at least in part, through novel non-canonical signalling pathways, we also tested the response of primary mammary epithelial cells in culture to signal through the smo-dependent canonical pathway. Under the conditions of primary cell culture, we observed that primary mammary epithelial cells did not induce expression of the Hh-target gene, mPtc1, and only weakly induced mGli1. In contrast, primary mesenchymal cells from the same animals responded robustly to the same N-Shh ligand, as evident by mPtc1 and mGli1 expression levels being strongly induced. The lack of signalling in primary epithelial cells through the canonical pathway is consistent with the lack of response we observed for human cell lines derived from breast cancers or transformed cells, including MCF7, MDA-MB-231 and MCF10A (Chang et al., 2010 and unpublished observations). For some of these latter lines, specifically MCF10A and MDA-MB-231, the expression of smo message appears to be absent, at least as determined by sensitive rtPCR studies of smo expression in these cells (Chang et al., 2010; Mukherjee et al., 2006; Zhang et al., 2009). The failure to stimulate the canonical pathway by N-Shh is distinct from the myriad of studies describing the consequences of altered expression of the downstream Hh-pathway components on cell growth, mobility, transformation or metastasis in mammary epithelial cell lines or, more often, breast cancer cell lines (for reviews, see Cho et al., 2013; Cui et al., 2010; Hui et al., 2013; O’Toole et al., 2009). However, these studies have typically not described the activation of the canonical Hh-pathway upon stimulation by Hh-ligand. Indeed, evidence suggests that the “Hh repression state”, that is the state in which Gli3 acts as a repressor (Gli3rep), is required for normal mammary gland development (Hatsell and Cowin, 2006; Hui et al., 2013). It might be expected, therefore, that changes to the expression of downstream factors that overcome the dominance of the Gli3rep might lead to altered cell characteristics and might contribute to cell transformation as occurs, for example, in the MMTV-Gli1 mice (Fiaschi et al., 2009). However, our data suggest further that, while the mammary epithelial cells appear refractory to Hh-signalling through the canonical pathway, non-canonical signalling cascades can be invoked.

To conclude, combined with our data characterizing the effect of the MMTV-c-srcAkt allele on development in the mes mammary gland, our data suggest that a novel Hh-signalling pathway involving the activities of ERα and c-src are required for mammary gland morphogenesis at puberty. These data suggest further that, for mammary epithelial cells, non-canonical pathways rather than the canonical Hh-pathway operating through smo are required for branching morphogenesis at this stage of mammary gland development.

Acknowledgements

This project was funded by a grant to PAH by the Canadian Institutes of Health Research (MOP-97929).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2014.04.007.

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