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

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ABSTRACT

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 smoothened, 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-src^{Act} 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, is 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.

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 regulates 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 Ser^{118} 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; Kousteni 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 Tyr^{416}, resulting in downstream activation of

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ras and MAPK (Migliaccio et al., 1998, 1996). The SH2-domain of c-src also interacts directly with ERα at Tyr^{537} (Arnold et al., 1995; Migliaccio et al., 2000; Sun et al., 2012). Phosphorylation of Tyr^{537} by c-src rapidly activates signal transduction pathways involving Erk and phosphoinositide 3-kinase (PI3K) (Varricchio et al., 2007), as well as stimulating ERα target gene transcription, ERα ubiquitylation and proteosomal degradation (Sun et al., 2012).

Genetic deletion of ERα in mice established important roles for this receptor for normal adult mammary gland development. Mammary glands of adult female ERα knock-out (αERKO) mice fail to respond to ovarian hormones resulting in a failure of ductal rudiments to elongate and fill the fat pad (Couse et al., 2000; 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 mPtch1, 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 ($c\text{-}src^{Act}$) 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-$c\text{-}src^{Act}$ 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/6N mice (Charles River) or C57Bl/6N mice heterozygous for the *mesenchymal dysplasia* (*mes*) allele of Ptch1 (JacksonLabs) were crossed with CERM mice, which harbour a flag-tagged ERα transgene under the control of the tet^{O} 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:

Mes: F 5′-TCCAAGTGTCGTCCGGTTTG-3′ and R 5′-GTGGCTTCCA-CAATCACTTG-3′ (Chang et al., 2012); FLAG-ERα: F 5′-CGAGCTCGG-TACCCGGGTCG-3′ and R 5′-GAACACAGTGGGCTTGCTGTTG-3′ (Miermont et al., 2010); MMTV-rtTa: F 5′-ATCCGCACCCTTGATGA CTCCG-3′ and R 5′-GGCTATCAACCAACACACTGCCAC-3′ (Miermont et al., 2010). Reaction conditions for MMTV-rtTa 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-rtTa 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. The glands were rinsed in water for 5 min and then stained in Carmine 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-Alx4 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 Ellipse fluorescent 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 (Capurro et al., 2012). Shh- and pcDNA-conditioned media were prepared by transfecting 40% confluent 10 cm plates of HEK293 cells in 10% FBS with 10 μg of pcDNA3.1-N-Shh (gift of J. Filmus, Sunnybrook Health Sciences Centre) or pcDNA3 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

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