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The molecular basis for progression of breast cancer from hormone dependence to hormone independence remains one of the critical issues in this prevalent but poorly understood disease. The principle objective of this proposal is to test the hypothesis that elevated stimulation of the c-Src kinase signaling pathway plays a critical role in modulating estrogen receptor function. The first specific aim of this proposal will assess whether the ductal outgrowth is due to the intrinsic inability of the mammary epithelial cell to respond to estrogen stimulation. To test this hypothesis we plan to assess whether introduction of exogenous estrogens can induce the c-Src deficient mammary epithelial cells to activate the MAP kinase pathway.

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## INTRODUCTION

The research objective outlined in the original proposal was to determine the significance of the ER $\alpha$  and c-Src interaction in mammary gland development and tumorigenesis. Earlier observations had suggested that estradiol stimulation of MCF-7 cells was associated with rapid transient tyrosine phosphorylation of the c-Src kinase and activation of downstream MAP kinase cascade (Migliaccio et al., 1996; Migliaccio et al., 1998). This transient tyrosine phosphorylation event was further correlated with the ability of c-Src and ER $\alpha$  to form specific complexes (Migliaccio et al., 1996; Migliaccio et al., 1998). To further explore the *in vivo* significance of this interaction, we examined the Src null mice for any obvious defects hormone regulated tissues such as the mammary gland. The results of these analyses revealed that in contrast to wild type siblings, the src null female mice exhibited a marked defect in ductal outgrowth and terminal end bud formation. As part of the preliminary data presented with this DOD application we further showed that the Src null epithelial cells lack the ability to tyrosine phosphorylate ER $\alpha$  following estradiol stimulation. Our first experimental goal was assess whether the mammary epithelial cells derived from the Src null mice exhibited defects in ER $\alpha$  coupled signaling pathways.

## BODY

1) The Src null mammary epithelial cells (MECs) exhibit defects in estradiol mediated activation of the MAP kinase, GSK3b phosphorylation, Akt kinase and STAT3 tyrosine phosphorylation.

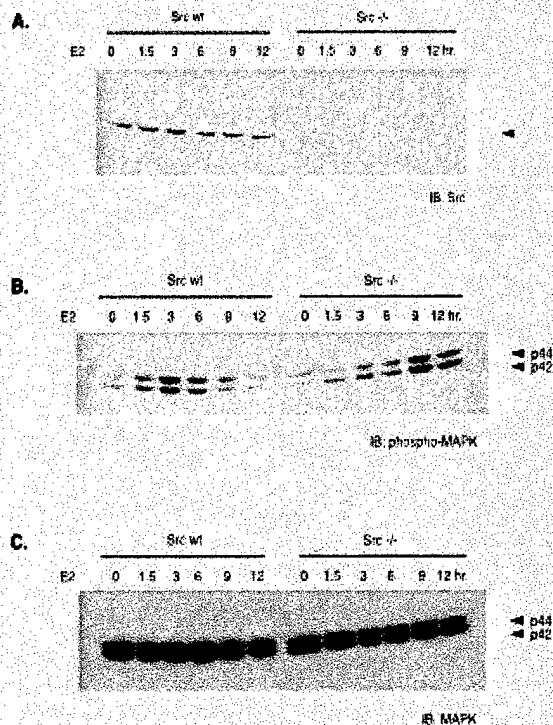


Figure 1. Absence of c-Src negatively impacts MAPK activation in the presence of E2.

(A) Mammary gland explants from wild type and *c-src*<sup>-/-</sup> mice were cultivated, serum starved and exposed to 100nM E2 over a defined time course, and extracts were subjected to immunoblot analyses with Src specific antisera (B) Upon E2 stimulation for the defined timepoints lysates were collected and subjected to immunoblot analysis with phospho-MAPK. (C) The levels of activated MAPK was not due to differences in total MAPK.

Figure 1

The major goal outlined in the original research proposal was to determine whether the MECs derived from the Src null mice exhibited defects in estradiol stimulation of the various signaling pathways. First, we examined whether MECs derived from the src deficient animals activated the Map kinase pathway in response to estradiol with similar kinetics to wild type

animals. As shown in Figure 1, primary src deficient MECs treated with estradiol displayed a delay in the kinetics of MAP kinase phosphorylation. Whereas the wild type MECs displayed maximal MAP kinase stimulation at 3 hours after estradiol administration, similar levels of MAP kinase activation were not achieved until 9 hours post-stimulation (Figure 1B). The difference in the kinetics of MAP kinase activation could not be attributed to differences in the levels of MAP kinase protein as both sets of samples contained comparable levels of MAP kinase (Figure 1C).

Another important signaling molecule that could potentially be influenced by estradiol stimulation of Src is the PI-3K/Akt kinase signaling axis (Castoria et al., 2001; Penuel and Martin, 1999) (Tsai et al., 2001). To explore whether activation of the Akt kinase could be influenced by estradiol stimulation, we subjected extracts derived from the estradiol stimulated

MECs to immunoblot analyses with phosphospecific antibodies directed Akt serine kinase. By contrast to wild type MECs where maximal stimulation of Akt occurred at 3 hours after estradiol stimulation, the Src null MECs exhibited a basal low level of Akt activation (Figure 2 A). The difference in the kinetics of Akt phosphorylation were not due to differences in the levels of Akt as both sets of samples expressed equivalent levels of Akt protein (Figure 2B)

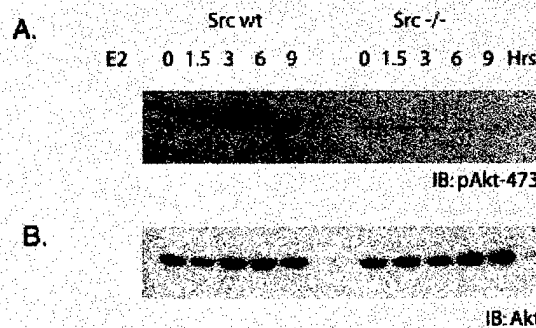


Figure 2: Loss of c-src attenuates Akt phosphorylation upon estrogen treatment of MECs. Mammary gland explants from wild type and c-src<sup>-/-</sup> mice were cultivated in CSS, serum starved and exposed to 100nM E2 over a defined timecourse. (A) MECs were lysed and probed with anti-Akt S473, a phosphorylation site that correlates with the activation of Akt. (B) Differences in phosphorylated protein levels were compared relative to total

mediated activation of Akt is the GSK-3 $\beta$  kinase. To assess whether phosphorylation of GSK-3 $\beta$

Another important signaling pathway that can be affected by Estradiol

was differentially regulated by MECs derived from either wild type or Src deficient MECs, protein extracts derived from estradiol treated MECs were subjected to immunoblot analyses

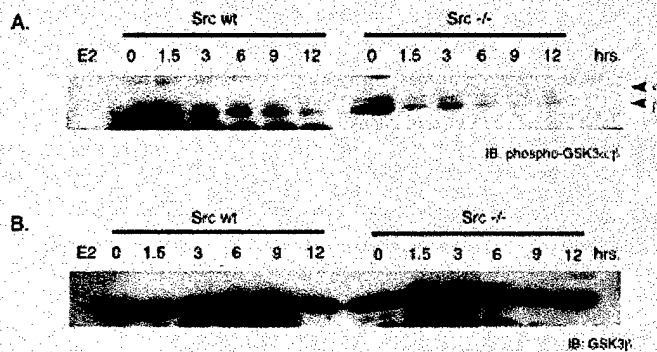


Figure 3. Effects of a loss of c-src on GSK3b function in MECs. Mammary gland explants from wild type and c-src<sup>-/-</sup> mice were cultivated in CSS, serum starved and exposed to 100nM E2 over a defined timecourse. (A) GSK3b activity was assessed by immunoblotting with a phospho-GSK3 antibody that detects both the a and b form of the protein. (B) Differences in GSK3 phosphorylation is not due to differences in total levels of GSK3b.

with phospho-specific GSK-3 $\beta$  antisera. The results revealed that the phosphorylation of GSK-3 $\beta$  peaked at 1.5 hours post-stimulation with estradiol in the wild type MECs. Phosphorylation of GSK3- $\beta$  was also sustained through the entire time-course of estradiol stimulation. In marked contrast, the Src deficient MECs demonstrated a dramatic decrease in phosphorylation of GSK-3 $\beta$  following estradiol stimulation (Figure 3A). The differences in the kinetics of GSK-3 $\beta$  phosphorylation could not be attributed to the levels of GSK since both sets of samples possessed equivalent amounts of GSK-3 $\beta$  (Figure 3B).

Another important downstream target of c-Src is the STAT-3 transcription factor (Garcia et al., 2001; Ren and Schaefer, 2002). To ascertain whether estradiol stimulation of Src could impact on tyrosine phosphorylation of STAT 3,

we performed immunoblot analyses on the MEC extracts with phosphospecific antibodies directed against STAT 3 tyrosine phosphorylation site 704. The results showed that following estradiol administration of the wild type MECs STAT3 was rapidly tyrosine phosphorylated peaking about three hours post-stimulation (Figure 4A). In src null MECs tyrosine phosphorylation of STAT 3 remained at basal level and eventually becoming de-phosphorylated at 6 hours post-stimulation (Figure 4A). To determine whether unrelated serine phosphorylation site was compromised we subjected the same extracts to immunoblot analyses with serine 727 phosphospecific antisera. In contrast to the tyrosine 704 phosphorylation, serine phosphorylation was unaffected by the loss of a functional c-Src protein. Again immunoblot analyses of the MECs extracts with STAT 3 antisera revealed comparable levels of STA3 a and b

isoforms. Taken together these observations argue that STAT 3 activation may be compromised by genetic ablation of c-Src. Given the documented importance of STAT3 in mammary gland development and tumorigenesis (Garcia et al., 2001; Ren and Schaefer, 2002), this in part may account for the observed defect in mammary gland development.

**(2 Transplantation of Src null epithelium into cleared mammary fat pads reveals a potential defect in the stroma**

Another important objective of the proposal was to assess whether the observed defect in ductal outgrowth was in part due to defective stroma. In this regard it is interesting

to note that in a similar ductal branching defect in the ER $\alpha$  mice is highly dependent on the stroma (Cunha et al., 1997). To test this possibility we transplanted wild type and src null epithelium into the cleared fat pads of syngenic FVB mice using standard transplantation techniques. After a three-week incubation period whole mount analyses was conducted on the mammary epithelium. The results revealed that the mammary epithelium derived from the Src null mice exhibited comparable extent of outgrowth as observed in the wild type epithelium (Compare Figure 5B and 5C). Untransplanted control cleared fat pads failed to exhibit any evidence of mammary epithelial outgrowth (Figure 5A). These observations suggest that in the context of wild type stroma, the Src null mammary epithelium can overcome any observed signaling deficit. These data also suggest that the Src null stroma is incapable of providing these compensatory signals. Future experiments involving the reciprocal transplantation of wild type mammary epithelium into Src null stroma should allow this issue to be addressed.

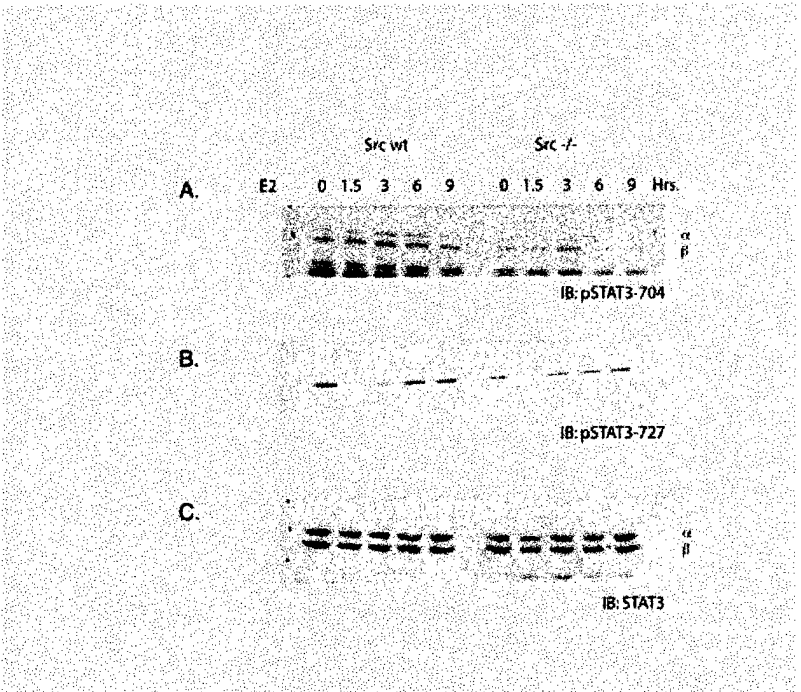


Figure 4: Effects of a loss of c-src on STAT3 function in MECs. Mammary gland explants from wild type and c-src -/- mice were cultivated in CSS, serum starved and exposed to 100nM E2 over a defined timecourse. (A) The ability of STAT3 to dimerize has been found to correlate with the phosphorylation of Y704. Phosphorylation of this residue was assessed by immunoblot analysis via a phospho-specific antibody that detects both the a and b versions of STAT3. (B) A similar analysis was done in order to assess the correlation between the loss of c-Src and the ability to transactivate via phosphorylation of S727. (C) Differences in phosphorylated proteins were compared to total levels of STAT3a or b.

**KEY RESEARCH ACCOMPLISHMENTS**

- Biochemical characterization of the src null MECs inability to respond to estrogen stimulation.
- Identification of a stroma defect in the c-Src null ductal outgrowth defect.

**Conclusions**

Although *in vitro* cell culture experiments suggest that formation of a c-Src/ER $\alpha$  complex is critical for estrogen action, the physiological importance of this interaction in the intact animal remains unclear. To further explore the *in vivo* significance between ER $\alpha$  and c-Src in normal mammary gland development, we performed a series of wholemount analyses of *c-src* deficient mice at various stages of mammary gland development. The results of these studies revealed that the female *c-src* null exhibited a dramatic defect in ductal outgrowth and terminal end bud formation that was further associated with a decrease in tyrosine phosphorylation of the ER $\alpha$  receptor. Furthermore, the primary mammary epithelium from *c-src* wild type mice displayed a rapid and sustained activation of the MAP in response to estrogen. In contrast, *c-Src* deficient mammary epithelium exhibited only a weak and transient response to MAP kinase stimulation. We further demonstrated that the estradiol treated MECs also displayed deficient response in number of signaling pathways downstream of c-Src including Akt, GSK-3b and STAT3 (Figures 2-4). The MEC and the MCF-7 data suggest that the *c-src* mammary gland defect is epithelial.

However, we also observe that reciprocal transplants point to a stromal component. The expression of c-Src has been documented to be ubiquitous, and taken together these observations are reminiscent of the ER $\alpha$  transplant data (Cunha et al., 1997).

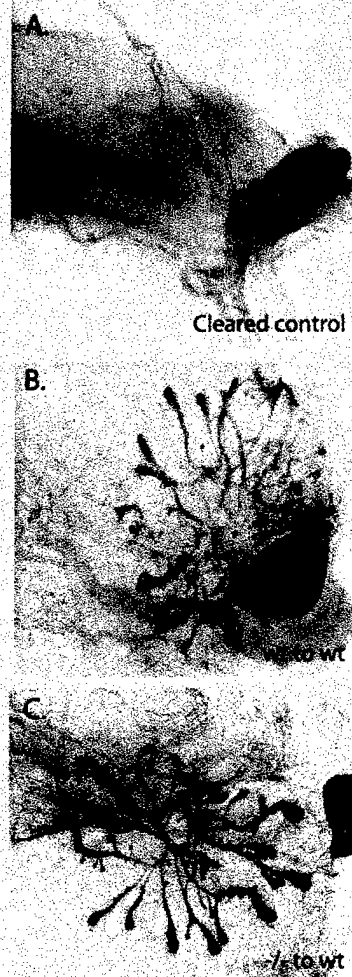


Figure 5: Transplants from *c-src* null to a wild-type cleared mammary gland suggest a stromal defect. The 4th inguinal gland of three-week-old virgin Fvb/n mice were cleared (A) and syngenic tissue from virgin female *c-src* null mice were transplanted. Whole mount analysis was performed 3 weeks post-surgery of wild-type controls (B) and *c-src* null (C) transplants. Magnification of 1.6x.

Superficially, the cell culture data and the transplant data appear to be incongruent, however this is not surprising. By definition, estrogen receptor studies in cell culture with pure populations of epithelial cells would never shed light on the interactions that occur between epithelium and stroma that leads to normal mammary gland development. Nevertheless, what is the mechanism within the stromal component that can compensate for the loss of c-Src and in turn a loss of ER $\alpha$  function in the epithelium? These factors may include the relative expression of ER $\alpha$  itself in each cellular compartment, the expression of other family members such as ER $\beta$ , differential expression of transcriptional cofactors in each cellular compartment or the presence of different upstream EREs that may define estrogen receptor function whether it is in the stroma or the epithelium. Alternatively the stromal related defect in ductal outgrowth may reflect defects cell functions unrelated to estrogen signaling. In this regard, the ductal outgrowth defect resembles a similar phenotype observed in the op/op mice which lack macrophages (Gouon-Evans et al., 2000).

Given these possibilities c-Src, and along the same line ER $\alpha$ , may either play a minimal role in the epithelium or a role that is not directly involved in ductal development. Evidence has shown that estrogen stimulation can induce EGF and EGFR expression in mammary tissues. Furthermore, reciprocal transplants utilizing EGFR null tissues demonstrate that EGFR function is absolutely necessary in the stroma to induce estrogen dependent ductal outgrowth (Wiesen et al., 1999). In light of the fact that c-ErbB2 can heterodimerize with the EGFR this places c-Src, ER $\alpha$  and EGFR/c-ErbB2 all within the stromal compartment of the mammary gland (Sebastian et al., 1998). Estrogenic conditions therefore appear to work in a paracrine fashion between the mesenchyme and the epithelium, estrogen activation being influenced by c-Src and MAPKs within the stroma, resulting in transactivation of ERE containing genes, notably EGF, that can influence epithelial cell growth and development.

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