# Dissertation

## A Single Nucleotide Polymorphism in the Steroid Receptor Coactivator 1 and its implications for estrogen dependent diseases

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# A Single Nucleotide Polymorphism in the Steroid Receptor Coactivator 1 and its implications for estrogen dependent diseases

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# Abbreviations

| | |
|---|---|
| AA | African American |
| Aa | Amino Acid |
| ACL | Acute Childhood Leukemia |
| AD | Activation Domain |
| AF | Activation Function |
| AI | Aromatase Inhibitor |
| AIB | Amplified in Breast Cancer |
| ALP | Alkaline Phosphatase |
| AP | Activator Protein |
| ATCC | American Type Culture Collection |
| BCM | Baylor College of Medicine |
| bHLH-PAS | Basic Helix-Loop-Helix-Per/ARNT/Sim |
| BMD | Bone Mineral Density |
| BMP | Bone Morphogenetic Protein |
| BRCA | Breast Cancer |
| CA | Caucasian American |
| CARM | Coactivator-associated Arginine Methyltransferase |
| CBI | Coactivator Binding Inhibitors |
| CBP | CREB Binding Protein |
| CI | Confidence Interval |

| | |
|---|---|
| COL1A1 | Collagen Type 1 Alpha 1 |
| COBRA | Consortium on Breast Cancer Pharmacogenomics |
| CREB | cAMP Regulatory Element-Binding Protein |
| CSS | Charcoal Stripped Serum |
| CYP | Cytochrome P 450 |
| DCIS | Ductal Carcinoma in situ |
| DKFZ | Deutsches Krebsforschungszentrum |
| DMEM | Dulbecco's Modified Eagle Medium |
| DXA | Dual-energy X-ray Absorptiometry |
| E2 | Estrogen |
| EGF | Epidermal Growth Factor |
| ER | Estrogen Receptor |
| ERE | Estrogen Responsive Element |
| ERK | Extracellular Signal Regulated Kinases |
| FasL | Fas Ligand |
| FasR | Fas Receptor |
| FBS | Fetal Bovine Serum |
| FGFR | Fibroblast Growth Factor Receptor |
| FHS | Framingham Heart Study |
| FRET | Fluorescence Resonance Energy Transfer |
| GLM | General Linear Model |
| GSK | Glycogen Synthase Kinase |
| GWAS | Genome Wide Association Study |
| H | Helix |

| | |
|---|---|
| HAT | Histone Acetyl Transferase |
| HEK | Human Embryonic Kidney |
| Het | Heterozygous |
| HRT | Hormone Replacement Therapy |
| IMEM | Improved Modified Eagle Medium |
| Ko | Knock-out |
| L | Leucine |
| LBD | Ligand Binding Domain |
| LRP | Lipoprotein-Receptor-Related Protein |
| M | Molar |
| MAPK | Mitogen-Activated Protein Kinase |
| MCF-7 | Michigan Cancer Foundation-7 |
| M-CSF | Macrophage- Colony Stimulating Factor |
| MEF | Mouse Embryonic Fibroblast |
| MMTV | Mouse mammary tumor virus |
| MTHFR | Methylentetrahydrofolate Reductase |
| MTS | (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) |
| NCOA | Nuclear Receptor Coactivator |
| NCOR | Nuclear Receptor Corepressor |
| NFκB | Nuclear Factor κ B |
| NKG | Natural Killer Cell Lectin-type Receptors |
| NR | Nuclear Receptor |
| NSABP | National Surgical Adjuvant Breast and Bowel Project Study |
| OPG | Osteoprotegerin |

| | |
|---|---|
| OR | Odd Ratio |
| Ovx | Ovariectomy |
| P | Proline |
| P53 | Protein 53 |
| PKA | Protein Kinase A |
| PMS | Phenazine Methosulfate |
| PPAR γ | Peroxisome proliferator-activated receptor |
| PR | Progesterone Receptor |
| PRMT | Protein Arginine Methyltransferases |
| PTH | Parathyroid Hormone |
| PTHLH | Parathyroid Hormone-Like Hormone |
| PTM | Posttranslational Modification |
| RANKL | Receptor Activator for Nuclear Factor κ B Ligand |
| Rs | Reference SNP |
| S | Serine |
| $^{35}S$ | $^{35}$Sulfur |
| SERM | Selective Estrogen Receptor Modulator |
| SMRT | Silencing Mediator for Retinoid and Thyroid Hormone Receptor |
| SNP | Single Nucleotide Polymorphism |
| SP-1 | Stimulatory Protein -1 |
| SRC | Steroid Receptor Coactivator |
| SUMO | Small Ubiquitin-related Modifier |
| T | Threonine |
| TGF | Transforming Growth Factor |

| | |
|---|---|
| TIEG | TGF-β Inducible Early Gene |
| TNF | Tumor Necrosis Factor |
| TPMT | Thiopurine S-Methyltransferase |
| TRAP | Tartrate Resistant Acid Phosphatase |
| TUNEL | Terminal deoxynucleotidyl transferase dUTP nick end labeling |
| UCSF | University of California San Francisco |
| VDR | Vitamin D Receptor |
| WISP | WNT1 Inducible Signaling Pathway |
| WNT | Wingless-type MMTV integration site family |
| WT | Wildtype |
| X | Any Amino Acid |

# 1 Background

## 1.1 Estrogen signaling

The steroid hormone estrogen is a key regulator of growth, differentiation, and the physiological functions of a wide range of target tissues[1]. Estrogen has a major role in the establishment and maintenance of reproductive function[2]. It plays also an important part for the skeleton, the cardiovascular system, nonreproductive centers of the brain[3], and cholesterol mobilization[4]. The majority of malignant breast cancers show a dysregulation in estrogen-dependent pathways resulting in enhanced mitogenic activity[2]. Estrogen acts through two receptors: estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ). Upon binding ligand, ER dimerizes and enters the nucleus where it interacts with a plethora of coregulators. ERα and ERβ are able to bind to the DNA through estrogen responsive elements (EREs) and can positively or negatively regulate gene transcription. ERα was also shown to activate transcription indirectly by binding to DNA binding proteins like stimulatory protein -1 (SP-1), c-fos, or c-jun[5]. Estrogen can act non-genomically to induce various signaling pathways, such as the Mitogen-Activated Protein Kinase (MAPK) pathway or calcium regulation[4,6].

### 1.1.1 The Estrogen Receptors ERα and ERβ

ERα and ERβ are members of the nuclear receptor family of ligand dependent transcription factors[7]. These receptors can bind to estrogen through their ligand binding domain (LBD) and regulate the expression of a large number of genes including signaling, cell cycle, and anti-apoptosis components[7].

Differential distribution of ERα and ERβ between tissues suggests that they have specific functions in specific tissues. A tissue-selective ratio of ERα/ERβ provides tissue-selective function[8]. Both receptors are usually expressed in the same tissue though ERα is the predominant subtype in breast and uterus, whereas ERβ is more highly expressed in the ovary and prostate[9,10].

ERα and ERβ display high sequence and structural similarities in their DNA-binding domain and moderate homology in their LBD (Figure 1).

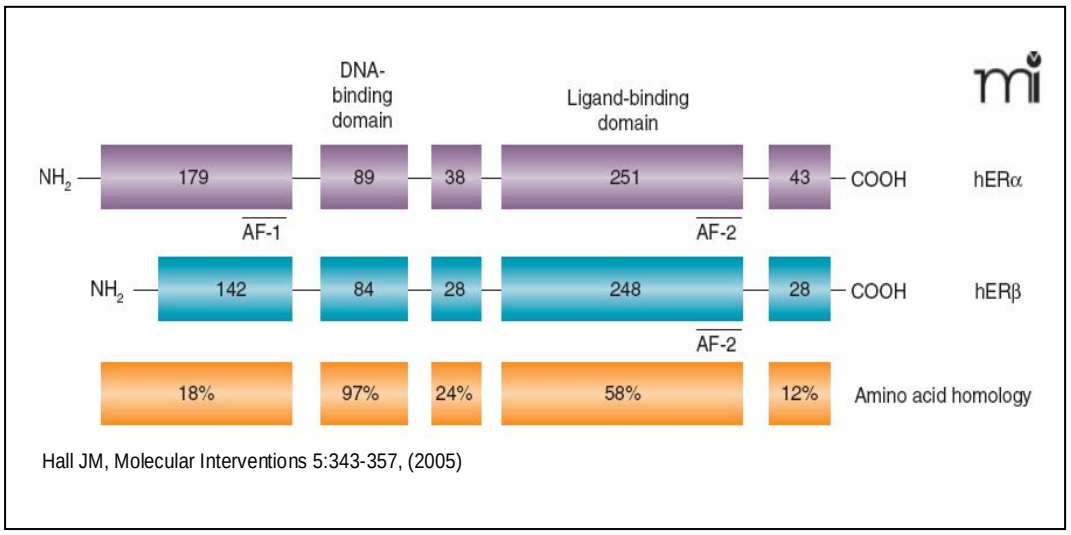


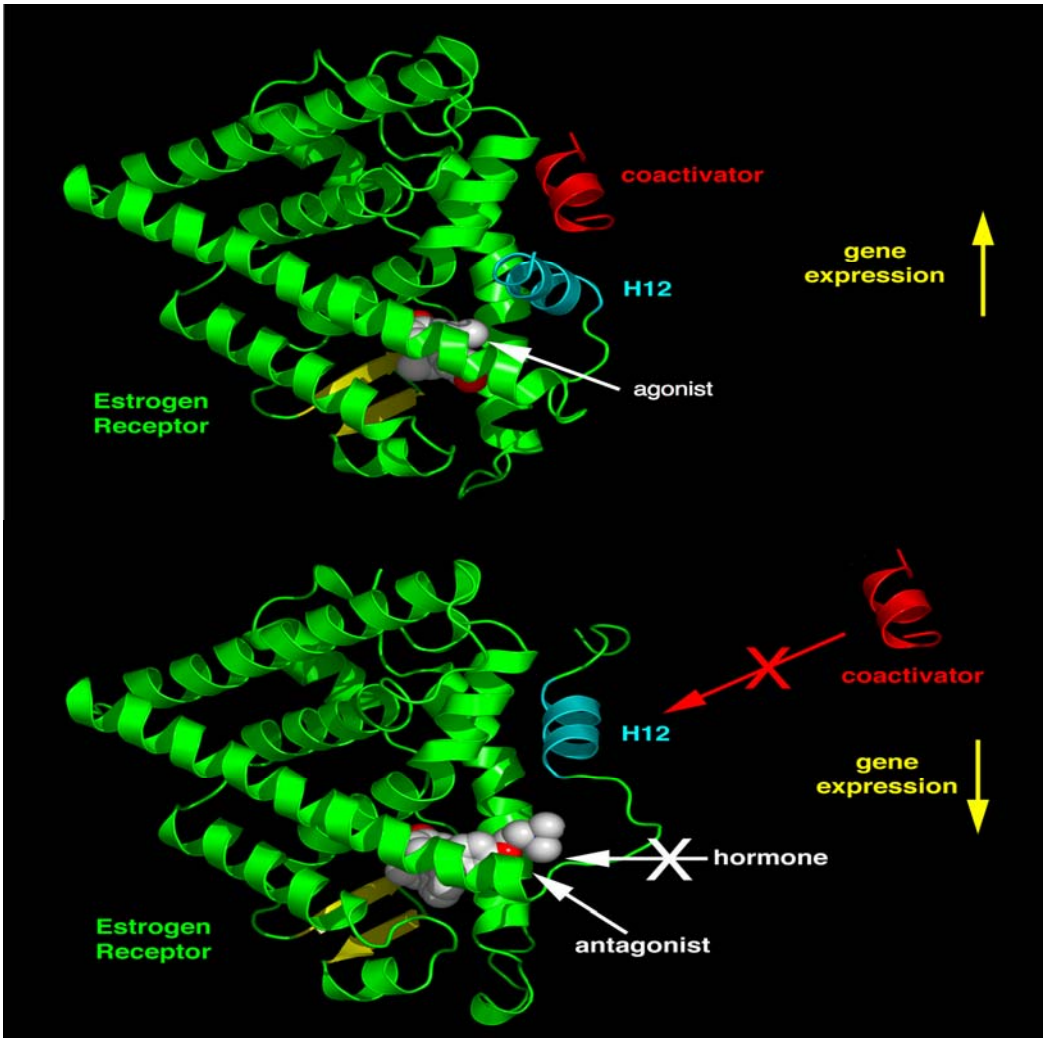

**Figure 1: Functional domains of the human estrogen receptors.**

**ERα and ERβ share a highly conserved central DNA-binding domain and moderately conserved C-terminal ligand-binding domain. The ligand-dependent transcriptional activities of the ERs are mediated through a C-terminal activation function (AF-2). ERα contains a constitutive AF-1 in the N terminus; no apparent AF-1 domain is present in the human ERβ. Numbers in boxes indicate the number of residues in each protein region[1].**

Due to their similarity, ERα and ERβ interact with similar DNA response elements and have similar binding affinities for estrogen[9,11].

ERs mainly exert their ligand-dependent transcriptional activity through a C-terminal activation function (AF-2) located in the LBD[1]. The main functional difference between the two receptors is determined by the difference in the AF-1 domain located in the N-terminus[4]. This determines ligand- and tissue- specific responses to estrogen (E2) and selective estrogen receptor modulators (SERMs)[4]. Most of the time those two AFs act synergistically, but it has also been shown that they can function independent of each other depending on cell type and promoter context[12]. AF-1 can perform its transactivating function in the absence of ligand[13]. Additionally, AF-1 is a target of the MAP-kinase pathway, indicating the possibility for cross-talk between growth factor- and estrogen-dependent pathways[14].

The LBD of ER and also other nuclear receptors (NRs) consist of 12 α-helices (H1-H12). In the presence of ligand the AF-2 pocket is formed by the folding of H12 against H3, H5/6, and H11[15] (Figure 2).




Brzozowski AM, Nature. 1997 Oct 16;389(6652):753-8

**Figure 2: Structural basis for the mechanism of nuclear receptor agonist and antagonist action[16].**

**The structures shown here are of the LBD of the estrogen receptor complexed with either agonist (top) or antagonist (bottom). The ligands are depicted as space filling spheres. When an agonist is bound to a nuclear receptor, the H12 is positioned such that a coactivator protein can bind to the surface of the LBD. Shown here is just the NR box containing the LXXLL (L- leucin, X- any amino acid) motif of the coactivator. Antagonists occupy the same ligand binding cavity of the NR. However, antagonist ligands in addition have a sidechain extension which sterically displaces H12 to occupy roughly the same position in space as coactivators bind. Hence coactivator binding to the LBD is blocked[16].**

Different ER ligands show different binding affinities to ERα or ERβ[17]. The binding of a true antagonist to ER changes the geometry of α-helix H12 which prevents subsequent binding of coactivators[4]. SERMs are a group of compounds that have agonist and antagonist properties for ER transcriptional activity. These compounds induce conformational changes in the ER LBD[18,19]. These changes result in recruitment of a specific subset of coactivators in different tissues, which might explain antagonistic properties in the breast and agonistic properties in the bone[18,19]. Even though coactivators like steroid receptor coactivators (SRCs) exert their hormone-dependent transcriptional NR coactivation by interacting with the AF-2 located in the LBD of NRs, they also interact weakly with the AF-1[20,21].

Both ERs can undergo posttranslational modifications (PTM). Phosphorylation by kinases like the MAPK and protein kinase A (PKA) were shown to enhance ER activity[22]. ER acetylation by histone acetyltransferases (HAT) can regulate ER transactivation and hormone sensitivity[23], and ubiquitinated ER can be targeted for degradation by the proteasome regulating ER levels and bioavailability[24, 25].

#### 1.1.1.1 Estrogen Receptor α and Estrogen Receptor β knock out mice models

The development of ERα and ERβ knock-out (ko) mice generated new insights in understanding its biological role. Male and female ERα ko mice are infertile[26, 27], proving ERα's importance for reproduction. Female ERα ko mice also showed deficient mammary gland development[26].

Contrary to ERα ko mice, mice lacking ERβ are fertile even though female ko mice produce significant smaller litters than wildtype (WT) mice. Moreover, female ERβ ko mice showed less differentiated mammary glands, what suggests a role for ERβ in breast cancer[28]. ERβ can act as a negative regulator for ERα when both receptors are expressed at the same time, mainly because it activates the same target genes but to a lesser extent[29]. Further, it shows inhibitory potential on the stimulation of cell proliferation by ERα[30]. Even though ERα is the predominant receptor in the uterus ERβ ko mice suggest that ERβ modulates ERα function in the uterus by conferring an antiproliferative function[31,32].

## 1.2 Estrogen Receptor - Coregulators

The extent and direction of ER activity is not only influenced by ligand binding to ER, but also by recruiting specific coregulators[33,34]. Enhancing nuclear receptor-mediated transcription activity by coactivators or inhibition by corepressors is needed for a balanced control of ER target gene expression[35]. Coregulators execute their function by

building high-molecular weight complexes consisting of multiple co-coregulatory proteins[36]. Coactivators are known to interact with ligand-bound NRs through their LXXLL motifs (L= leucine, X= any amino acid) and enhance the receptor target gene transcription, while corepressors bind to unliganded or ligand bound nuclear receptors and mediate transcriptional repression of target genes. The cellular levels of coregulators are determined by PTM[36]. They are targets for degradation by ubiquitin-dependent and -independent mechanisms. Degradation can be inhibited by phosphorylation of certain sites resulting in higher cellular levels[8]. A substantial amount of work has been done characterizing the role of ERα-coactivator complexes in transcriptional activation of a large number of genes. However, less is known about the functions of ERα-corepressor complexes in transcriptional repression despite the fact that they play a key role in breast cancer prevention[37]. The most studied coregulators are the SRC family, nuclear receptor corepressor 1 (NCOR1) and the silencing mediator for retinoid and thyroid hormone receptor/ nuclear receptor corepressor 2 (SMRT/ NCOR2). These factors have been detected in various cell and tissue types. An insight into the role of these cofactors was achieved by generating specific ko mice and determining the resulting phenotype.

### 1.2.1 Coactivators

Coactivators bind with their LXXLL motif containing NR interaction domain to the hydrophobic groove on the surface of the NR LBD in the presence of agonist. Recruitment of coactivators to the ER-agonist complex leads to activated gene transcription by altering chromatin architecture, loosening the nucleosome structure, and activating RNA polymerase II[1]. Even though the LXXLL domain is highly conserved between different coactivators the amino acids surrounding this motif determine binding affinity as well as specificity to interact with NRs[38, 39]. Surrounding amino acids of identical LXXLL motifs can determine the recruitment of a different combination of coactivators and therefore result in different gene responses[8]. Differential recruitment of NR coactivators causes tissue specificity of SERMs resulting in either antagonisitic activity in the breast or agonistic activity in the uterus[40]. Activation of coactivators can occur by PTM such as phosphorylation or mono-ubiquitination[41, 42]. Within the active coregulator complex PTM by acetylation and methylation regulate protein-protein interaction and inactivation can occur by sumoylation and poly-ubiquitination[8,42].

#### 1.2.1.1 The p160 Steroid Receptor Coactivator family

The SRC family contains three homologous members (SRC-1, SRC-2, and SRC-3) that are known to enhance NR mediated transcription from target genes through their

LBD[16,43]. SRCs are known to interact with several NRs as well as other transcription factors like activator protein-1 (AP-1)[44], nuclear factor-κB (NFκB)[45], and cAMP regulatory element-binding protein (CREB)[46]. Binding of SRC to a transcription factor initiates recruitment of other chromatin modifiers such as the acetyltransferases CREB binding protein (CBP) and p300 and the methyltransferases coactivator-associated arginine methyltransferase 1 (CARM1) and protein arginine methyltransferase 1 (PRMT1) and activate transcription of their target genes[47,48]. SRC family members contain multiple similar functional domains (Figure 3).

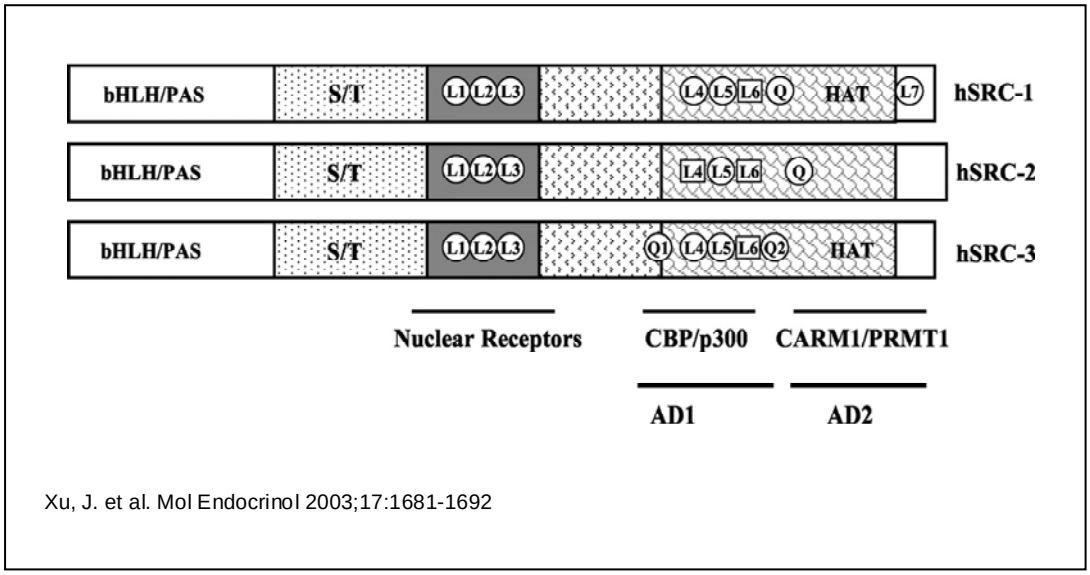



**Figure 3: Structural and Functional Domains of the SRC Family Members[49].**

**The similarity and identity of amino acid sequences for full-length human SRC proteins and their specific conserved regions are indicated above the bars. The letters within the bars indicate structural domains, and the lines under the bars indicate domains that interact with different factors or serve as transcriptional activation domains (AD) 1 and 2. PAS, Per/ARNT/Sim homologous domain; S/T, serine/threonine-rich regions; L, LXXLL -helix motifs[50]; Q, glutamine-rich regions; HAT, histone acetyltransferase domains identified in SRC-1 and SRC-3[51,52].**

Their N-terminal basic helix-loop-helix-Per/ARNT/Sim (bHLH-PAS) domain is the most conserved region among SRC family members[53]. The bHLH-PAS domain can serve as a DNA-binding or protein-protein interaction surface for various bHLH-PAS-containing factors[54]. They contain conserved LXXLL motifs to bind the NR LBD, which contains the AF-2[16,43]. Two intrinsic transcriptional activation domains (AD1 and AD2) are located in the C-terminal receptor interaction domain of the SRC molecules[55]. The AD1

contains multiple LXXLL motifs that are responsible for interactions with the HAT CBP and p300[55]. The C-terminal domains of SRC-1 and SRC-3 possess weak HAT activities[52]. HATs allow chromatin remodeling at target promoters and facilitate transcriptional activity[2,52]. The AD2 can interact with protein arginine methyltransferases (PRMT), such as CARM1 and PRMT1[47,56]. Based on protein structure, SRCs may mainly serve as adaptor proteins to recruit additional coactivators to the promoter[57].

SRCs are expressed at similar levels and exhibit similat binding affinities to ER, suggesting that specific regulation of coactivators in specific tissues is important for their function[19]. MAPK-mediated phosphorylation can activate SRC-1 and SRC-3[58,59] and SRC-2 is more active in the presence of the methyltransferase CARM1[47].

Even though it was shown that SRC-1, SRC-2, and SRC-3 can partially compensate for one another[60], they have different functions in different tissues[49]. Selective recruitment of SRCs by different NRs may determine the specific assembly of coactivator complexes to mediate specific transcription signals[61]. Variable tissue-specific expression patterns of SRC family members may also be responsible for their functional specificities[49].

#### *1.2.1.1.1 Steroid Receptor coactivator 1 (SRC-1)*

SRC-1 [nuclear receptor coactivator 1 (NCOA1)] was first identified *in vitro* by its ability to interact with progesterone receptor (PR) and subsequently to enhance NR activity in a ligand-dependent manner[62]. The generation of the SRC-1 ko mouse[63] provided insight into the role of SRC-1 in estrogen-dependent tissue. Mice lacking functional SRC-1 protein exhibited normal growth, fertility, and normal female reproductive behaviors[64] but showed resistance to steroid hormones[63]. The estrogen-induced uterine growth and the estrogen- and progesterone-dependent uterine decidual response were decreased in ovariectomized female SRC-1 ko mice[49]. During development, the SRC-1 ko mice may adapt a genetic compensatory mechanism such as up-regulation of other coactivators for maintenance of fundamental biological functions. Indeed, the level of SRC-2 mRNA is slightly elevated in the brain of SRC-1 ko mice[63], suggesting a mechanism of genetic compensation from other SRC family members during development.

*In vitro* and *in vivo* data suggest a role for SRC-1 in breast cancer. SRC-1 interacts with ERα and ERβ. Mammary gland ductal side branching and alveolar formation was reduced in ovariectomized female SRC-1 ko mice treated with estrogen and progesterone[49]. Importantly, in human SRC-1 expression is positively correlated with HER2 status, resistance to tamoxifen treatment, and tumor recurrence[65]. SRC-1 is negatively associated with disease free survival and positively associated with breast tumor size[66]. Moreover,

immunostaining for SRC-1 revealed expression in the nuclei of breast tumor epithelial cells but not in normal breast tissue[66]. Collectively, the aforementioned data on SRC-1 strongly supports its crucial role in breast cancer progression and endocrine treatment efficiency.

The overexpression of the SRC-1 in osteoblastic cell lines resulted in preferential transcriptional enhancement by ERβ, whereas SRC-2 overexpression appeared to preferentially enhance ERα transactivation[67]. Differential recruitment and expression levels of SRC coactivators might explain the observed differences in ER activation[67].

SRC-1 male and female ko mice develop osteopenia with high bone turnover in the trabecular bone, but not in the cortical bone[68]. The consequences of SRC-1 deficiency for estrogen action on bone *in vivo* were characterized[69]. Recognizing that SRC-1 ko mice have compensated estrogen resistance[63], Moedder et al. not only characterized the skeletal phenotype under basal conditions but also after ovariectomy (ovx) and replacement with a dose of estrogen that was found to be the minimal physiological dose necessary to prevent bone loss in female[69] but not in male mice[70]. Under basal conditions female SRC-1 ko mice exhibit normal skeletal phenotype and bone mineral densities (BMDs)[69]. However, by ovariectomizing the animals and using a dose of estrogen that was effective in preserving BMD in the WT mice, they were able to unequivocally demonstrate a profound defect in estrogen action on cancellous bone in the SRC-1 ko mice[69]. Moreover, this defect could be overcome by using a 4-fold higher dose of estrogen, consistent with skeletal estrogen resistance[69]. In contrast to this marked deficit in estrogen action in cancellous bone, the effects of estrogen on cortical bone in the SRC-1 ko mice were relatively well preserved[69].

#### *1.2.1.1.2 Steroid Receptor coactivator 2 (SRC-2)*

SRC-2 ko mice exhibit nearly normal somatic growth[49], but their fertility is significantly reduced in both male and female mice[49]. Male hypofertility is due to a decrease in sperm number, defective maturation of the spermatid acrosome, and age-dependent testicular degeneration[49]. The decreased female fertility is due to placental hypoplasia caused by the absence of maternal SRC-2 in decidual stromal cells that face the developing placenta[71]. This indicates that SRC-2 plays a critical role in reproductive behavior and functions. Further it was shown that SRC-2 plays an important role in lipid metabolism and energy balance[72]. In the white adipose tissue, SRC-2 serves as a coactivator for Peroxisome proliferator-activated receptor γ (PPARγ)[72].

SRC-2 is expressed in many tissues including the mammary gland, but to date it has no relevant association to breast cancer[49].

#### *1.2.1.1.3 Steroid Receptor coactivator 3 (SRC-3)*

The third member of the SRC family, SRC-3 is expressed in many tissues including the mammary gland. SRC-3 ko mice display growth retardation, reduced adult body size[73,74], diminished female fertility, and a delay in mammary gland growth[73]. Estrogen levels are significantly lower in female SRC-3 ko which causes a delay in pubertal development[73]. Additionally, mammary gland alveolar development in response to a combined stimulation of estrogen and progesterone was significantly decreased in adult SRC-3 ko females, suggesting that SRC-3 is involved in hormone-dependent cell proliferation and glandular differentiation during breast alveolar development[73].

Implying its importance for breast cancer it is also called AIB1 (amplified in breast cancer). SRC-3 is overexpressed in 60% and amplified in up to 10% of breast tumors[53] and its overexpression correlates with ER positivity and tumor size[75].

Depletion of SRC-3 in MCF-7 breast cancer cells significantly reduces the estrogen-mediated cell proliferation and inhibition of apoptosis[49]. Down-regulation of SRC-3 in MCF-7 cells also reduces estrogen-dependent colony formation in soft agar and tumor growth in nude mice[76]. Furthermore, mice that overexpress SRC-3 specifically in the mammary gland developed malignant mammary tumors[77]. In human breast tumors SRC-3 protein expression is linked to high tumor grade[78].

### 1.2.2 The role of posttranslational modifications (PTMs) for SRCs

PTM is the chemical modification of a protein after its translation. PTM of amino acids extends the range of functions of the target protein by attaching to it other biochemical functional groups such as phosphate (phosphorylation), acetyl (acetylation), methyl (methylation), ubiquitin (ubiquitination), or the small ubiquitin-related modifier (SUMO) called sumoylation.

PTM of ER and cofactors were shown to be important for either enhancing or decreasing transcriptional coactivity or to regulate assembly or disassembly of ER- coregulator complexes[1].

#### 1.2.2.1 Phosphorylation

Phosphorylation of SRCs leads to conformational changes at distinct sites, precludes the formation of surface binding sites for other proteins[79], and consequently may enhance or

inhibit their activity[80]. Phosphorylation sites identified in SRCs are targets of kinase-mediated signaling pathways such as the MAPK pathway, the cAMP/PKA pathway, and the NF-κB pathway[81].

Major phosphorylation sites of SRC-1 are consensus sites for the serine/threonine-proline-directed family of protein kinases[58]. Two identified phosphorylation sites contained a consensus sequence for the MAPK family[58]. Phosphorylation of those sites in vitro can affect SRC-1 activity[58]. In the absence of hormone phosphorylation of SRC-1 by the cAMP/PKA pathway enhances the interaction of SRC-1 with CBP/p300 for optimal activation of ligand-dependent and ligand-independent transcription of PR[58].

One phosphorylation site has been identified in SRC-2, which potentiates SRC-2's coactivity[82]. Epidermal growth factor (EGF)-induced MAPK pathway phosphorylation of this site enhances SRC-2 interaction with CBP/p300[79]. Phosphorylation of SRC-2 by the cAMP/PKA pathway causes down-regulation and degradation of SRC-2[83].

There are six phosphorylation sites in SRC-3 which are all required for ER coactivation. Different combinations are required for the activation of NF-κB or for oncogenic transformation of mouse embryonic fibroblasts (MEFs)[84]. Phosphorylation of SRC-3 by MAPK activates SRC-3's intrinsic acetyltransferase activity and enhances the recruitment of CBP/p300[59]. SRC-3 can be phosphorylated by the NF-κB pathway in an ER-independent fashion resulting in increased NF-κB transactivation and immune-inflammatory responses[85].

#### 1.2.2.2 Acetylation

Acetylation of SRCs plays a role in the repression of coactivator signaling[86]. Acetylation of SRC-3 leads to disassociation of coactivator- receptor complexes and subsequently compromised transcriptional activity[81].

#### 1.2.2.3 Methylation

Methylation of SRCs is involved in transcriptional repression[87,88]. All SRC family members are substrates for CARM- dependent methylation, but only SRC-3 has been extensively studied[87,88]. Methylation of SRC-3 can be induced by estrogen signaling and regulates the stability of SRC-3 by increasing its degradation[87,88].

#### 1.2.2.4 Ubiquitination

Ubiquitin is a highly-conserved regulatory protein that is ubiquitously expressed in eukaryotes. Ubiquitination refers to the PTM of a protein by the covalent attachment of

one or more ubiquitin monomers. Addition of a single ubiquitin is a reversible process that can be rapidly switched off by deubiquitylating enzymes and is a crucial regulator of the activity and transportation of cellular proteins[89,90]. Addition of a long polyubiquitin chain becomes irreversible and triggers degradation of proteins via the ubiquitin-proteasome pathway[91]. SRCs are targets for ubiquitin-dependent degradation[81]. Mono-ubiquitination of SRC-3 can increase the proteins activity, whereas poly-ubiquitination catalyzes the proteins degradation[42]. The AD2 of SRCs may be accountable for ubiquitination- dependent processes since the AD2 of SRC-2 is essential for proteasome degradation[92].

#### 1.2.2.5 Sumoylation

All three SRC family members can be sumoylated[81]. SUMO proteins are a family of small proteins that are covalently bound to other proteins and subsequently modify their function in cells. Most SUMO-modified proteins contain the tetrapeptide consensus motif Ψ-K-x-D/E where Ψ is a hydrophobic residue, K is the lysine conjugated to SUMO, x is any amino acid (aa), D/E is an acidic residue. SUMO proteins are similar in structure to ubiquitin, and Sumoylation is directed by an enzymatic cascade analogous to that involved in ubiquitination. In contrast to ubiquitin, SUMO is not used to tag proteins for degradation. Sumoylation of SRC-1 or SRC-2 was shown to increase their coactivator potential by retaining the protein in the nucleus[48,93]. In contrast, sumoylation of SRC-3 can decrease its transactivty[94].

## 1.3 Breast cancer

Breast cancer is the most frequent cancer among women to date. Two thirds of breast cancer tumors are ERα positive[95], suggesting that estrogen is a strong risk factor for the initiation and progression of breast cancer. Breast cancer occurs more frequently when ERα is over-expressed in adjacent normal epithelium[96]. ER status is a prognostic factor for breast cancer and a predictive factor for response to endocrine therapy[97]. ERα is an important clinical target for endocrine therapy and ER positive breast cancer prevention. Further, understanding of its mechanism of action can lead to improvements in drug development. Although, ERα is the abundant form of ER expressed in breast tumors, ERβ was reported to be expressed in 30-70% of breast cancers[65,98,99]. The role for ERβ in carcinogenesis has yet to be determined; however the protein might have inhibitory effects on cellular proliferation[100]. Contrary to ERα, ERβ expression does not correlate

with tumor grade[4]. However, the ERα/ERβ ratio appears to be associated with tumor progression[101].

### 1.3.1 Breast cancer treatment

Two thirds of all breast tumors are ER positive. Selective treatment of those cancers is possible by blocking estrogen stimulated growth.

#### 1.3.1.1 Antiestrogens

Drugs exerting antiestrogen potential are divided into pure antiestrogen, which act as an ER antagonist in all tissues like ICI 182,780 (Faslodex). Faslodex completely inhibits the assembly of an active transcriptional complex at the ERE[75].

SERMs show tissue-selective antagonist-agonist properties[102-104]. SERMs currently approved for the treatment of breast cancer and osteoporosis are tamoxifen (Figure 4), raloxifene, and toremifene. Since its synthesis in 1975, tamoxifen, a partial/selective ERα antagonist, is the preferred standard treatment for ERα positive breast tumors.

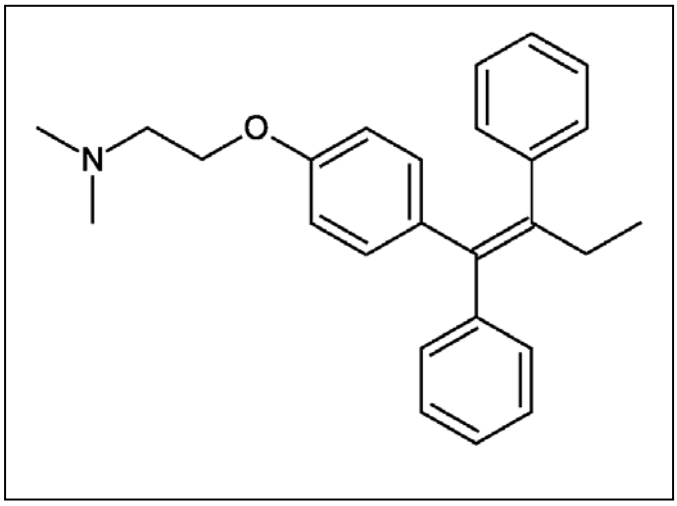



**Figure 4: Structure of tamoxifen.**

Treatment with tamoxifen has been shown to improve disease-free and overall survival[105] and to induce remission in ER positive metastatic breast cancers[106,107]. Additionally, tamoxifen was shown to have a protective effect and decreasing the risk of developing ERα-positive breast cancer[4]. Tamoxifen shows agonistic properties in the bone and can therefore prevent postmenopausal bone loss, however, exerts its agonistic potential in the uterus resulting in an increased risk of endometrial cancer[102-104]. Notably, premenopausal women treated with tamoxifen do not develop higher risk for endometrial cancer or blood clots[108]. The second generation SERM raloxifene is approved for treatment and

prevention of osteoporosis[109,110] and to reduce breast cancer incidence in high risk postmenopausal women[111]. Raloxifene shows the same potential in preventing and treating breast cancer as tamoxifen without increasing the risk of endometrial cancer[109,110]. Toremifene has shown the same agonistic potential in the uterus as tamoxifen and is only used in postmenopausal women with metastatic breast cancer[4]. Differential recruitment of coactivators and corepressors is a major factor in determining if SERMs act as an antagonist or agonist[35]. Genes that are activated by tamoxifen bound ERα are different than genes that are activated by estrogen bound ERα[4]. Tamoxifen-bound ERs are recruited to different promoters what can again result in regulation of different genes[40].

#### 1.3.1.2 Aromatase inhibitors (AI)

AIs are a class of drugs used in the treatment of breast cancer and ovarian cancer in postmenopausal women that block the aromatase enzyme and therefore the biosynthesis of estrogen[112] (Figure 5).

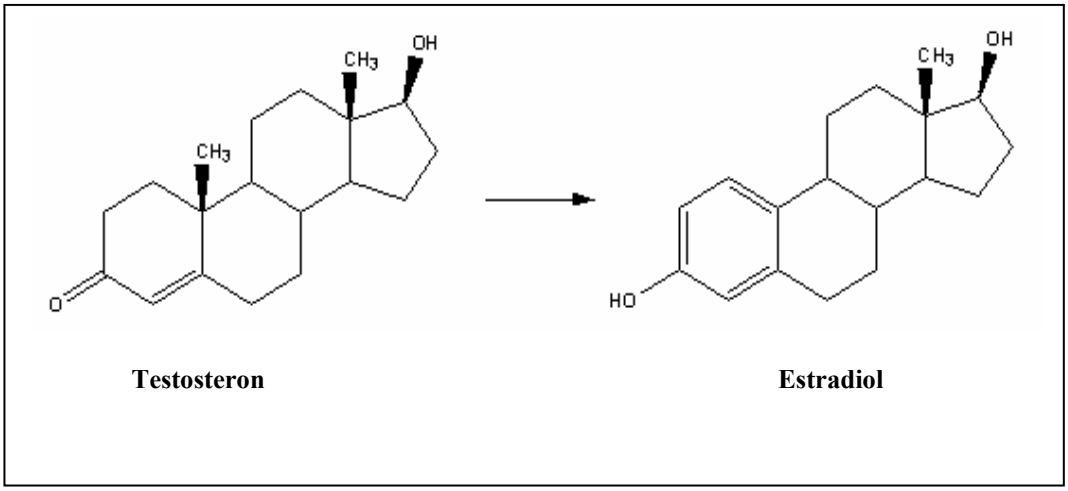



**Figure 5: Aromatase converts testosterone to estradiol**

AIs can be divided into irreversible steroidal inhibitors such as exemestane which form a permanent bond with the aromatase enzyme complex and non-steroidal inhibitors (anastrozole, letrozole), which inhibit the enzyme by reversible competition[113]. In premenopausal women most of the estrogen is produced in the ovaries, while in postmenopausal women estrogen mostly is produced in the adrenal gland from the conversion of androgens[114]. Since most of the circulating estrogen is produced by the ovaries, not by conversion of androgens to estrogen, blocking the enzyme aromatase does not significantly decrease the production of estrogen. Therefore, AIs are generally not used to treat breast cancer in premenopausal women. When aromatase inhibitors are used in premenopausal women, the decrease in estrogen activates the hypothalamus and pituitary axis to increase gonadotropin secretion, which stimulates the ovary to increase androgen production. This counteracts the effect of the aromatase inhibitor[114]. As adjuvant treatment for postmenopausal women AIs show advantages over tamoxifen towards developing blood clots or endometrial cancer and improved efficacy[115,116]. However, Tamoxifen remains the preferred treatment for premenopausal women and ductal carcinoma in situ (DCIS)[117].

#### 1.3.1.3 Coactivator binding inhibitors (CBI)

CBIs are a new group of small molecules that inhibit the interaction of ER with coactivators like members of the SRC family directly[118]. They are designed to bind to the hydrophobic groove of the ER-agonist complex and block interaction between ER and SRCs[118]. Due to the suggested direct block of the protein-protein interaction, resistance as observed with antiestrogens may not occur. A structure-guided approach was taken to

identify cyclic systems that display three hydrophobic substituents and therefore mimic the three leucine residues in the LXXLL interaction motif between ER and coactivators[118]. This approach identified 2,4-diamino-6-alkyl pyrimidines as the target structure[119]. In time-resolved fluorescence resonance energy transfer assays (FRET) and cell based assays of estrogen transcriptional activity CBIs can block ER activity and this effect can not be reversed with increasing amounts of estrogen[118]. The most potent compounds are selective for ERα over ERβ. While CBI's still need to be approved and studied in clinical settings, they might provide an alternative approach to endocrine therapy and resulting resistance.

### 1.3.2 Tamoxifen resistance

Unfortunately, the majority of breast cancer patients treated with tamoxifen develops resistance within 5 years of treatment[120,121]. Tamoxifen can stimulate the growth of hormone-resistant breast cancer tumors[122].

Raloxifene appears to be an alternative to the widespread use of tamoxifen. In studies treatment of ER positive breast cancer with raloxifene for eight years was found to be effective in 65% of all breast cancer cases[109]. This suggests that some tumors may become raloxifene resistant[109]. Cross-resistance between tamoxifen and raloxifene has been reported[123]. Nevertheless, tamoxifen is the most prescribed endocrine therapeutic drug for pre-menopausal and post-menopausal women to date.

Tamoxifen is a prodrug that mainly conducts its full antiestrogenic and antitumor activity by biotransformation to endoxifen[124,125]. The main enzyme that is involved in this biotransformation is cytochrom P 450 (CYP) 2D6[126]. Ten percent of the population carries CYP2D6 variations which can influence tamoxifen's metabolism and result in decreased treatment efficacy[127].

Since most tamoxifen resistant tumors still express functional ERα, tamoxifen resistance is due to a mechanism other than loss of ERα[128]. Further, those tumors remain responsive to growth inhibition by pure antiestrogens like ICI 182780 and other hormonal therapies[129]. Tamoxifen resistance might be a result of switching the antagonist into an agonist[130-132]. The known intrinsic estrogenic activities of tamoxifen in bone or uterus can explain the acquired resistance in breast tumors[133].

Coactivators, like members of the SRC family, enhance the agonistic properties of tamoxifen in specific tissues[20,40]. This observation leads to the conclusion that tamoxifen resistance could result due to a change in coregulator levels. Protein expression analysis

of SRC-1 showed that initially estrogen treatment resulted in increasing levels of SRC-1 whereas tamoxifen treatment caused a decrease as measured in 30% of primary breast cell cultures derived from patient tumors[65]. Expression of the co-activator SRC-1 is higher in uterine Ishikawa cells, where tamoxifen functions as an agonist, than in MCF-7 breast cancer cells, where it functions as an antagonist[40]. Overexpression of SRC-1 in MCF-7 cells converted tamoxifen to an agonist, while decreasing SCR-1 expression in Ishikawa cells converted tamoxifen to an antagonist[40]. These findings suggested that tamoxifen resistance in breast tumors might be due to overexpression of SRC-1 and/or other co-activators, leading to the conversion of tamoxifen from an antagonist to an agonist.

SRC-1 expression is associated with resistance to endocrine therapy in breast tumors[66] and with the growth factor receptor HER-2[66]. Enhanced growth factor stimulation is known to determine the efficiency of breast cancer treatment with antiestrogens[134,135]. Intrinsic tamoxifen resistance is associated with HER-2, ER positivity, and PR negativity in tumors with increased SRC-3 levels[136]. This suggests that SRC coactivators, especially SRC-1 and SRC-3 may serve as predictive factors for tamoxifen response.

## 1.4 Osteoporosis

Osteoporotic bone loss is the result of high bone turnover in which bone resorption outpaces bone deposition[137,138]. Osteoporosis is one of the most common disorders in the elderly and it affects approximately 40% of postmenopausal women[139]. Multiple pathogenic mechanisms are the cause for progressive bone loss[140]. As a result the major complications of this disease are vertebral and hip fractures causing high rates in morbidity and mortality[141]. To identify individual fracture risk generally the BMD by dual-energy X-ray absorptiometry (DXA) is determined[142]. Low BMD is considered to be the strongest risk factor for fractures[143].

### 1.4.1 Bone structure

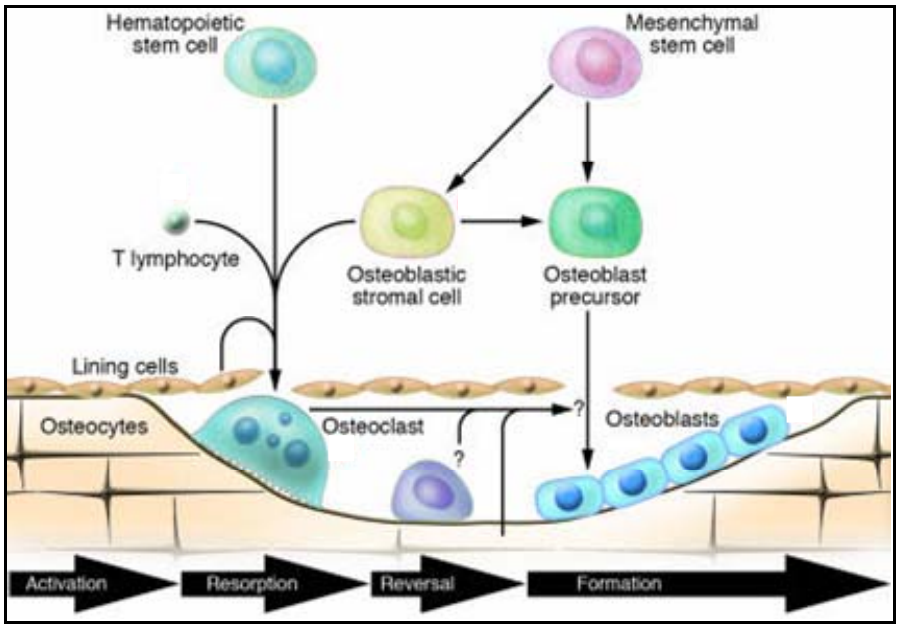
The outer layer of bones is composed of cortical bone tissue and accounts for 80% of the total bone mass of an adult skeleton[144]. Trabecular bone (also called cancellous bone) displays the interior of bone which is composed of a network of rod- and plate-like elements that make the overall organ lighter and allowing room for blood vessels and marrow[144]. Trabecular bone accounts for the remaining 20% of total bone mass, but has nearly ten times the surface area of cortical bone[144]. There are three main types of cells constituting the bone. Osteoblasts are mononucleate bone-forming cells, which descend

from osteoprogenitor cells[144]. They are located on the surface of osteoid seams and make a protein mixture known as osteoid, which mineralizes to become bone[144]. Osteoid is primarily composed of Type I collagen[144]. Osteoblasts also manufacture hormones, such as prostaglandins, which act on the bone[144]. Osteoblasts produce matrix proteins and alkaline phosphatase, an enzyme that has a role in bone mineralization[144]. Osteocytes originate from osteoblasts and become surrounded by bone matrix which they themselves produce[144]. The spaces which they occupy are known as lacunae. Osteocyte's functions include formation of bone, matrix maintenance, and calcium homeostasis[144].

Osteoclasts are the cells responsible for bone resorption. Osteoclasts are large, multinucleated cells, derived from hematopoietic stem cells in the bone marrow[145]. They are located on bone surfaces creating a shallow resorption pit known as a "Howship's lacuna"[144]. Because the osteoclasts are derived from a monocyte stem-cell lineage, they are equipped with phagocytic like mechanisms similar to circulating macrophages[144]. Osteoclasts migrate to discrete bone surfaces. Upon arrival, active enzymes, such as tartrate resistant acid phosphatase (TRAP), are secreted to digest the mineral substrate[144].

### 1.4.2 Bone remodeling

Bone remodeling on the surface of trabecular bone is a process that involves the bone resorption by osteoclasts and a formation phase by osteoblasts (Figure 6)[140]. This process is similar to bone remodeling in the cortical bone.




Raisz LG, J Clin Invest. 2005 December 1; 115(12): 3318–3325

**Figure 6: Schematic presentation of bone remodeling[140]**

**Hematopoietic precursor cells become activated osteoclasts by interacting with cells of osteoblastic lineage. The resulting activated osteoclasts initiate a limited bone resorption phase. During the following reversal phase the bone surface is covered by mononuclear cells, which then initiate the stimulation of osteoblasts to start bone formation[140].**

### 1.4.3 Regulation of the bone remodeling process

#### 1.4.3.1 The role of estrogen and its receptors in bone

Estrogen is a key hormone in bone remodeling in several species. The osteoprotective action of estrogen is demonstrable in rodents and is clinically important in humans, particularly in older women[137,140,141,146]. Postmenopausal women are at high risk for osteoporosis due to estrogen deficiency. Postmenopausal women develop a decrease in cortical as well as trabecular BMD[147]. Estrogen deficiency increases bone resorption rather than decreasing bone formation[148,149]. Demonstrating the ctritical role of estrogen in the pathogenesis of osteoporosis, estrogen treatment reduces bone loss in postmenopausal women[150]. Estrogen can directly act on osteoblasts and osteoclasts and alter either bone formation or bone resorption, by affecting cellular differentiation, proliferation, or regulating target gene expression[139]. Estrogen also induces apoptosis in osteoclasts[151].

Osteoblasts, osteoclasts, and osteocytes express ERβ as well as ERα[152]. In the trabecular bone ERβ is more expressed than ERα[153,154], whereas ERα is the predominant receptor in cortical bone[152]. Some studies suggest the two receptors exert the opposite effect on bone, whereas others suggest similar agonistic properties[155,156], with ERα being the major mediator for bone remodeling[157].

The development of specific ER knockout mice has proven useful in understanding estrogen action in the skeleton. For a knockout of ERα in mice, exon 3 of ERα was deleted[158], whereas the described ERβ and ERα/β ko mice still express a truncated form of ERβ[159]. Phenotypically female and male ERα ko mice show a decrease in cortical BMD, as well as decreased bone formation[26]. The deletion of the ERβ gene in female mice results in increased longitudinal bone growth and increased BMD[156,160,161]. These data suggest that ERβ may exert a negative effect on ERα-mediated bone growth in mice. In contrast to the described mouse models a natural mutation of ERα in human, which inactivates the receptor, results in continued longitudinal bone growth[162]. The double ko mouse for ERα and ERβ shows a decrease in cortical and cancellous bone suggesting the two receptors can substitute partially each others function[159]. Also these mice indicate that ERβ plays a role for bone maintenance distinct from ERα. The observed phenotype of both ERα/β mice mimics bone loss observed in postmenopausal women.

Osteoclast specific ERα ko mice show clear trabecular bone loss and high bone turnover associated with increased osteoclast numbers in females but not in males[163]. There is no decrease in cortical BMD[163] contrasting the ERα ko mouse phenotype[26]. These findings suggest that ERα is not only important in osteoclasts but also in osteoblasts in maintaining BMD, but the differences in phenotypes still need to be examined.

#### 1.4.3.2 Estrogen regulated genes in bone

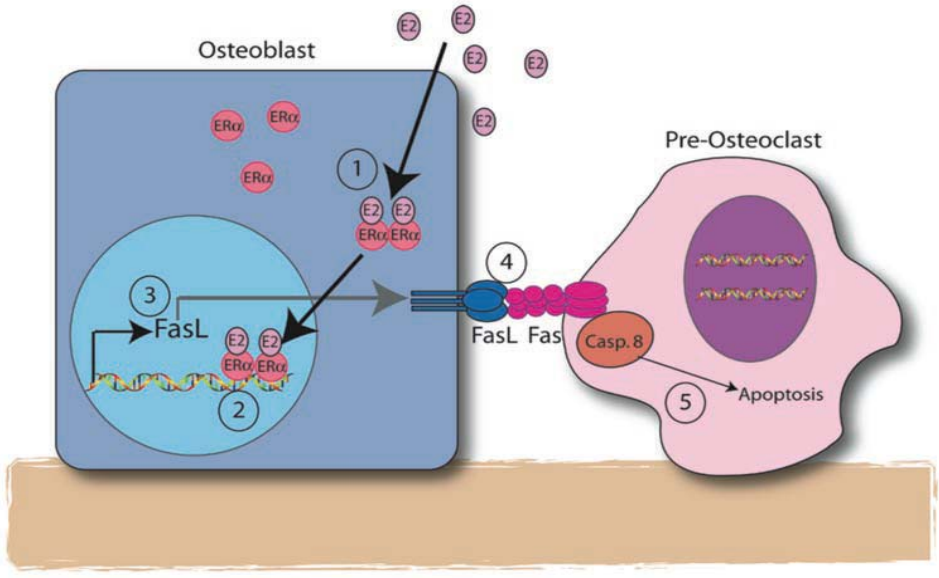
ERα and ERβ are both expressed in bone. The identification of regulated genes allows insight into the pathways and gene networks regulated by estrogen through these two ERs[164]. Estrogen regulates genes encoding cytokines, growth factors, and bone matrix proteins in trabecular bone of mice[161]. Genes encoding proteins associated with the regulation of the immune response such as members of the Natural Killer cell lectin-type receptors (NKG family) were upregulated by estrogen[164]. Another category of genes regulated by estrogen in bone were associated with regulation of cell motility and the cytoskeleton[164]. Most of the estrogen regulated genes involved in cytoskeleton regulation and motility, signal transduction, cytokine, and immune response and growth factors/hormones were commonly regulated through both receptors[164]. Important growth

factors and hormones regulated by estrogen through both receptors include several genes that can act in either an autocrine or paracrine manner to affect the bone-protective actions of estrogen[165]. These genes include the Wnt1 inducible signaling pathway -2 (WISP-2), a connective tissue growth factor isolated from osteoblasts[166], the bone morphogenetic protein 6 (BMP6), a potent osteogenic factor for the bone-protective actions of estrogens, and the parathyroid hormone-like hormone (PTHLH), a homolog of the parathyroid hormone (PTH), that acts as an anabolic agent in osteoporosis[167]. Several genes are regulated specifically by ERα or ERβ in the presence of estrogen[164]. Osteoclastogenesis inhibitory factor is specifically downregulated in the presence of ERα. Recent studies identified Fas Ligand (FasL) as an ERα regulated gene that is upregulated in response to estrogen to induce osteoclast apoptosis[163,168]. In contrast the TGF-β inducible early gene (TIEG) is upregulated in response to estrogen by recruiting ERβ[169] and has been associated with osteoblast differentiation[170].

#### *1.4.3.2.1 Fas Ligand*

FasL is a type II transmembrane protein that belongs to the tumor necrosis factor (TNF) family. The binding of FasL with its receptor Fas Receptor (FasR) induces apoptosis. Estrogen upregulates the expression of the FasL gene and increases apoptosis in differentiated osteoclasts in WT female mice. This effect was undetectable in mice lacking osteoclastic ERα[163]. Estrogen as well as SERM induction of FasL and apoptosis requires ERα in cultured osteoclasts[163]. The osteoprotective actions of estrogen and SERMs are mediated in part through osteoclastic ERα in trabecular bone, and the life span of mature osteoclasts is regulated through the activation of FasL signaling. Cortical bone mass is increased in ovariectomized osteoclast specific ERα ko female mice during estrogen treatment, the antiresorptive estrogen action in cortical bone may be mediated by osteoblastic ERα. FasL induction by estrogen in osteoblasts may contribute to the osteoprotective estrogen action, and FasL gene induction by estrogen was in fact detected in primary cultured osteoblasts from female calvaria[168].

Estrogen induces FasL in osteoblasts to subsequently induce apoptosis in osteoclasts in a paracrine manner (figure 7).




Krum, S. A. et al. EMBO J 2008;27:535-545

**Figure 7: Estrogen upregulates FasL in osteoblasts to induce apoptosis in osteoclasts[168].**

**(1) E2 binds to ERα in the cytoplasm followed by nuclear localization of ERα**

**(2) ERα binds to an enhancer element downstream of the FasL transcriptional start site**

**(3) FasL transcription is induced**

**(4) FasL binds to the FasR on the pre-osteoclast surface**

**(5) Activation of FasR leads to cleavage of caspase 8 and subsequent apoptosis.**

Purified osteoblasts, the osteoblast derived cell line MC3T3, and stably overexpressing ERα osteosarcoma cell line U2OS mRNA levels of FasL are upregulated after treatment with estrogen[168]. Osteoclasts do not undergo estrogen induced apoptosis unless osteoblasts are added in a co-culture experiment and that ERα in osteoblasts is sufficient to induce osteoclast apoptosis. Additionally, SERMs such as tamoxifen and raloxifene are also able to induce FasL expression in osteoblasts and stimulate osteoclast apoptosis.

Estrogen deficiency following menopause or ovariectomy leads to high bone turnover, particularly in the trabecular areas, as bone is rapidly lost through enhanced

resorption[141,171]. Thus, estrogen treatment leads to recovery from osteopenia by reducing resorption[137,141], partly by the induction of osteoclast cell death.

#### *1.4.3.2.2 Transforming Growth Factor β inducible early gene- 1 (TIEG)*

TGF-β is an important autocrine and paracrine factor for bone formation and maintenance, which was originally cloned from human osteoblasts[170]. TIEG is a member of the Sp/Krueppel-like transcription factor family, which are involved in antiproliferative and apoptosis inducing functions similar to TGF-β[172]. TIEG is rapidly and transiently induced in osteoblasts within 60 minutes of TGF-β treatment[173].

TIEG ko mice show an osteopenic phenotype characterized by decreased bone content, density, and size in trabecular as well as cortical bone[170]. Bones of TIEG ko mice have a significant reduction in osteocytes, suggesting that defects in the osteoblast differentiation exist and may be responsible for the observed defects in bone morphology and strength[170]. An increase in the number of osteoblasts, without a subsequent increase in bone formation parameters[174] suggests further that osteoblasts derived from TIEG ko mice are unable to fully differentiate and mineralize in culture[174]. TIEG deficient osteoblastic cell cultures show decreased expression of important osteoblast differentiation markers like alkaline phosphatase and osteocalcin[174].

Estrogen has been shown to induce TIEG-1 expression in human osteoblasts[175]. This estrogen induction is regulated by recruiting ERβ but not ERα to a regulatory region in intron 1 of TIEG[169]. The AF-1 of ERβ is responsible for recruiting SRC-1 to this regulatory region, which may be essential for TIEG induction by ERβ[169].

### 1.4.4 Treatment of osteoporosis

Current treatment of osteoporosis is focused on reducing the risk of fractures by preventing bone loss[141]. The imbalance in bone turnover that is induced by estrogen deficiency in women can be ameliorated with bio-available estrogens including SERMs[176].

#### 1.4.4.1 Hormone Replacement Therapy (HRT)

HRT is used to supplement the body with either estrogen alone or estrogen and progesterone in combination during and after menopause. The initial study showed that estrogen is very effective in reducing bone loss as well as decreasing fracture risk also showed a significantly increased risk of breast cancer and cardiovascular disease[177]. HRT is also associated with a 30% increased risk of ovarian cancer[178]. Since risks exceed the

benefits of this therapy approach it is mainly abandoned as the primary therapy, but led to the common use of SERMs to treat osteoporosis.

#### 1.4.4.2 Selective Estrogen Receptor Modulators

Raloxifene has been shown to decrease fracture risk without increasing the risk for breast cancer[179]. To compare the relative effects and safety of raloxifene and tamoxifen on the risk of developing invasive breast cancer and other disease outcomes, the National Surgical Adjuvant Breast and Bowel Project Study (NSABP) of Tamoxifen and Raloxifene was conducted (STAR trial)[180]. This prospective trial indicated no difference in osteoporotic fractures after treatment with either tamoxifen or raloxifene. However, raloxifene showed a reduced risk for developing endometrial cancer compared to tamoxifen. It has been suggested that raloxifene has to be administered for at least 10 years to maintain effective treatment of osteoporosis[8].

#### 1.4.4.3 Bisphosphonates

Bisphosphonates decrease bone loss and fracture risk[179,181]. Currently approved by the FDA and the Bundesinstitut für Arzneimittel und Medizinprodukte (BfArM) are alendronate, risedronate, and ibandronate. They act by binding to the bone surface, are absorbed by osteoclasts and cause subsequently inactivation and apoptosis of these cells. Extended use of bisphosphonates is concerning, since they can cause decreased bone remodeling resulting in slowing down the process of repairing fractures and micro damages[182].

## 1.5 Single nucleotide polymorphisms (SNPs)

With a number exceeding ten million[183], SNPs are the most common genetic variations in the human genome[184]. SNPs are defined as substitutions of a single base pair with a minor allele frequency higher than 1% in a normal population[185]. At least 1 in 1000 basepairs displays this kind of genetic alteration[186]. One important aspect to be considered when performing functional *in vitro* and *in vivo* studies on SNPs is that SNPs located at nearby sites are not inherited randomly. They build haplotype blocks, as a set of SNPs that are statistically associated. It is thought that these associations, and the identification of a few alleles of a haplotype block, can unambiguously identify all other polymorphic sites in its region. Linkage disequilibrium analysis as a measurement for the non-random association of these alleles can be employed to infer the haplotypes[184].

The majority of SNPs don't contribute to a change in the amino acid sequence of a protein (synonymous SNPs), or occur in non-coding regions. However, in some SNPs (nonsynonymous SNPs) the nucleic acid change results in an altered amino acid. These genetic alterations are of interest as they may confer a predisposition to disease or may affect treatment efficacy. Studying these SNPs may lead to the development of more individualized treatment strategies. SNPs in various genes contribute to a change in risk, outcome, or treatment efficacy for several diseases.

Two different approaches can be utilized to identify SNPs in genes that confer to risk for developing a disease or for differences in treatment efficacy.

The candidate gene approach investigates SNPs in target genes implicated in the disease being studied. To study the impact of SNPs towards therapeutic response, candidate genes are selected based on signaling and metabolic pathways[187]. This approach can reduce the risk of false-positive findings that can occur in genome wide association studies (GWAS), but has the disadvantage of possibly excluding genes important for disease and treatment reponse[187].

GWAS as an examination of genetic variations across the whole genome identified SNPs in genes that are associated with disease and were not previously known to be target genes for those diseases[187]. These studies gave new insights into pathophysiology and pharmacology of the disease examined. Due to the completed HapMap project, easy accessible genomic databases, and the development of high-throughput automated genotyping, increasing numbers of GWAS linking SNPs to diseases are available. As mentioned above, a disadvantage of these studies is the possibility of a large number of false-positive associations. Further, since GWAS can be cost-intensive, functionally interesting SNPs or haplotype-tagging SNPs to represent a gene can be used for analysis more economically[187].

### 1.5.1 SNPs associated with cancer

In cancer studies SNPs can influence either susceptibility or outcome. Several SNPs in target genes have been identified to confer to differences in risk or outcome for several types of cancers.

Variants in the thiopurine S-methyltransferase (TPMT) can result in severe haematopoetic toxicity[188]. The protein encoded by TPMT catalyzes the S-methylation of thiopurines, which are used to treat haematopoietic malignacies and autoimmune disorders. Thiopurine drugs are activated to cytotoxic thioguanine nucleotides.

Biotransformation of the drug includes methylation by TPMT leading to inactivation of the drug. In the presence of a genetic variant in TPMT resulting in decreased activity, severe toxicity was observed. Children harboring genetic variants resulting in inactivation of TPMT are at greater risk for relapse in acute childhood leukemia (ACL). Types and frequencies of TPMT variations vary among ethnic groups[189,190]. Overall it was estimated that 71% of patients who showed intolerance to thiopurines were TPMT deficient[191]. Appropriate dose reduction for those patients resulted in similar toxicity and survival outcome[192,193]. Testing for TPMT is now used before the initiation of thiopurine therapy[194].

#### 1.5.1.1 SNPs associated with breast cancer

5-9% of all breast cancer cases are inherited[195]. Only 30% of those cases show mutations in Breast Cancer (BRCA) 1 and BRCA2, which are known to contribute to a high risk of developing breast cancer[196]. This suggests that additionally low penetrance variations, alone or in combination, in breast cancer susceptibility genes can account for a higher risk in developing breast cancer[197]. Those genes could be involved in DNA repair, steroid hormone metabolism and signaling, and carcinogen metabolism[198].

Associations with polymorphisms and various breast cancer phenotypes have been identified. Genetic variations in CYP1A1, CYP1B1, BRCA1, and protein 53 (p53) are associated with differences in breast cancer risk, ER and PR status, and lymph node status[197]. The identified variations were already reported earlier to be associated with breast cancer susceptibility[199-204]. SNPs in the EGFR kinase domain are able to increase receptor activity resulting in pharmacodynamic changes in response to the EGFR tyrosine kinase inhibitor gefitinib[205].

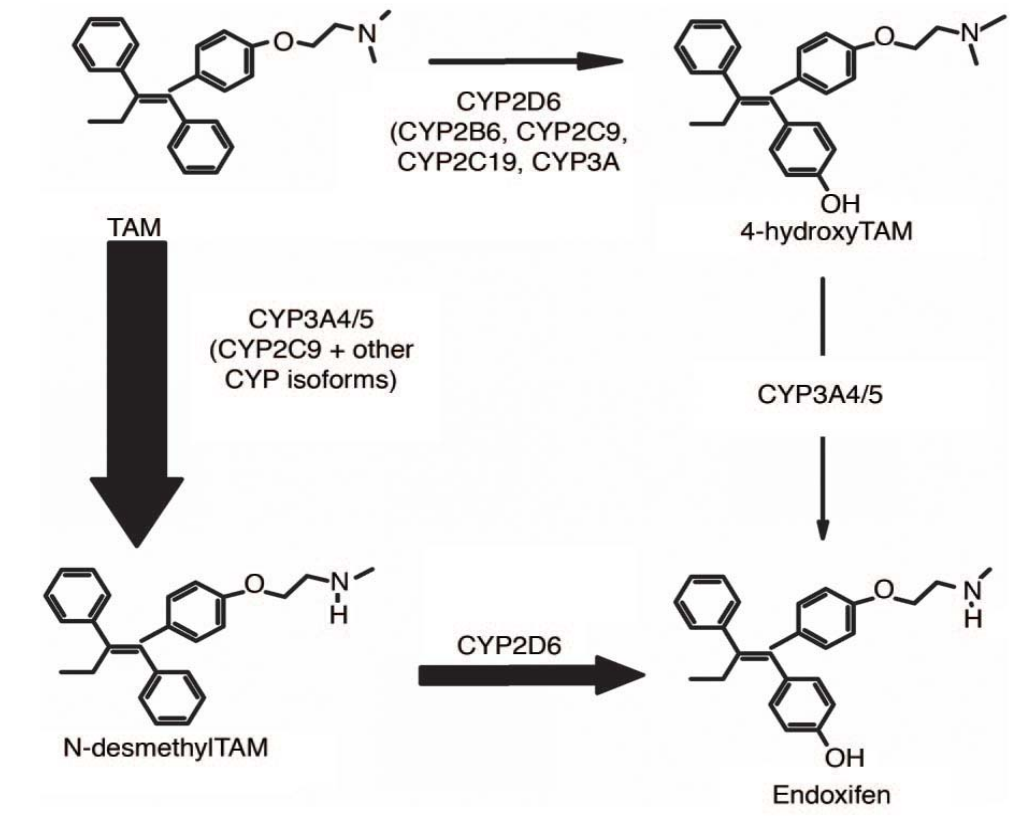
Recent GWAS identified genes resulting in increased risk for breast cancer[206,207]. Most highly associated SNPs with the disease are located in genes that were not initially expected to be related to breast cancer from the perspective of a candidate gene approach. The majority of disease-associated variations are located in introns making it more difficult to elucidate how those changes contribute to the disease. Both studies have identified the fibroblast growth factor receptor 2 (FGFR2) as risk factor for breast cancer[206,207]. In Europeans, the allele that predisposes individuals for breast cancer is inherited as a haplotype of eight SNPs located in intron 2 of FGFR2[206,207]. FGFR2 was previously shown to be important for mammary gland development[208]. Meyer *et al.*[209] described how two of those changes might increase the risk to develop breast cancer. The two variations they studied are able to alter the binding of two transcription factors

important for mammary gland-specific gene expression[209]. Resulting in an increase of FGFR2 expression, which was previously shown to be associated with ER-positive breast cancer[210].

#### *1.5.1.1.1 SNPs in CYP2D6*

SNPs can predict severe adverse effects, therefore, confer to treatment efficacy, and indicate individuals which should not receive a certain drug. SNPs can also predict risk for developing certain diseases[194].

Prediction treatment efficacy studies have focused on variants in the CYP450 system. Specifically, SNPs in CYP2D6 can alter the activity of CYP2D6 and the pharmacokinetics of anticancer drugs[211]. CYP2D6 is the major enzyme which transforms tamoxifen into its more potent and abundant metabolite endoxifen (figure 8). SNPs in CYP2D6 can effect plasma concentration levels of tamoxifen metabolites and determine response and toxicity to the drug[212]. Endoxifen, like 4-hydroxytamoxifen, has 100-fold greater affinity for ER and a 30 to 100 fold greater suppression in estrogen-dependent cell proliferation[213]. Endoxifen shows a 10-fold higher abundance than 4-hydroxytamoxifen, supporting that endoxifen is even more important for the anticancer effect of tamoxifen[212].




Goetz, M. P. et al. J Clin Oncol 2005; 23:9312-9318

**Figure 8: Selected transformation pathways of tamoxifen and the main CYP enzymes involved.**

**The relative contribution of each pathway to the overall oxidation of tamoxifen is shown by the thickness of the arrow, and the principal P450 isoforms responsible are highlighted in larger fonts[212].**

The genotype CYP2D6*4 is associated with poor tamoxifen metabolism[212]. Women treated for breast cancer with tamoxifen, which were homozygous for this genotype (CYP2D6 *4/*4) have decreased relapse free and disease-free survival if compared to heterozygous or wildtype (WT) for this allele[212]. This suggests that a decrease in CYP2D6 activity can increase the risk of tamoxifen treatment failure. To support the importance of sufficient metabolism of tamoxifen to endoxifen, women carrying two alleles of CYP2D6*4 did not experience hot flashes as side effects for tamoxifen therapy if compared to heterozygous or WT CYP2D6[212].

#### *1.5.1.1.2 SNPs in coregulators*

It is important to study SNPs in coregulators as very little is known about how they contribute to altered breast cancer, osteoporosis risk or estrogen-dependent treatment efficiency. Characterization of genetic variation in ER coregulators will lead to a better understanding of how the genetic background influences the development of breast cancer and osteoporosis and the efficiency of treatments.

A recent case-control study by Burwinkel *et al.*[214] focused on two SNPs in SRC-3 that were not linked to each other. They identified a significant breast cancer protective effect for a nonsynonymous SNP at amino acid position 586 that causes an amino acid change from glutamine to histidine. Glutamine586 is located 35 amino acids upstream of the first LXXLL motif that is needed to bind nuclear receptors and is hypothesized to alter the structure of SRC-3 thus decreasing its activity. Additionally, a synonymous SNP at amino acid position 960 was also shown to have a significant breast cancer protective effect due to the usage preference for one codon over another. Although, this study has described relevance of these SRC-3 SNPs to breast cancer, the functional consequences have not been examined.

### 1.5.2 SNPs associated with osteoporosis

Osteoporosis is a common disease that was shown to include a strong genetic component with 50-70% heritability as shown from family linkage studies[215-217]. Since osteoporosis is known to be a disease involving multiple pathogenic mechanisms, SNPs in target genes involving pharmacological and metabolical pathways are likely to exceed small but clinically important effects[140,218]. So far more than 30 candidate genes have been reported to potentially influence bone mass and fragility[219,220].

The human aromatase CYP19 catalyzes the conversion from testosterone to estradiol[218] and is therefore important for estrogen-mediated bone maintenance. Polymorphisms in CYP19 have been studied extensively. However, the results vary to associated changes with osteoporosis related markers[218,221-223]. The polymorphism has been associated with BMD and vertebral fracture risk[218,221], modification of BMD response towards HRT[218,222], and circulating levels of estrogen in postmenopausal women[218,223]. The differences in results might be explained with different ethnicity or age distribution within each studied population.

In addition, a recent GWAS for heritable traits that contribute to fracture risk was conducted in the Framingham Heart Study (FHS)[142] and identified one SNP in CYP19.

Several other SNPs in osteoporosis candidate genes like ERα, low density lipoprotein-receptor-related protein 5 (LRP5), vitamin D receptor (VDR), collagen type 1 alpha 1 (COL1A1), and methylentetrahydrofolate reductase (MTHFR) have been identified to be associated with osteoporosis[142]. Polymorphisms in those genes can still explain only a small percentage of the variation in BMD and fracture risk[224-227] and the described associations need to be replicated in additional studies.

Another recent GWAS for associations with BMD, osteoporosis, and osteoporotic fractures also identified a SNP in LRP5 to be associated with BMD and osteoporotic fractures[228]. Although the two different GWAS identified two different SNPs in LRP5, they are not considered to be in linkage disequilibrium with each other. This supports that the LRP5 locus might be an interesting region for influencing BMD and fracture risk. The other SNP identified by Richards et al.[228] that showed genome-wide association with BMD was located near the osteoprotegerin (OPG) gene. A GWAS conducted in Icelandic subjects for association with BMD and fracture risk showed that SNPs in the genomic region of OPG, and ERα are most strongly associated with those phenotypes[229].

Altogether, genetic variations predicting for disease risk or drug response are intensively studied and very common, but only a few are incorporated into clinical practice to improve individual treatments so far. Reasons for that are that most associations cannot be sufficiently replicated in different studies due to sample size, ethnicity, age distribution or other confounding factors.

Recent GWAS on associations with BMD and fracture risk indicate that there are several genomic loci such as ERα, LRP, and OPG where SNPs frequently occur and result usually in associations with bone related phenotypes, thus, identifying important genes for the pathology of osteoporosis. Those specific changes vary within different studies within those loci, making it necessary to conduct more studies to identify functional variants. This further supports the knowledge that most genetic variatons show low to medium penetrance and account only for a small part to explain interindividual differences in risk or drug response. Therefore, it is more likely that a list of genetic variations together exert the potential to identify the right drug and dose response for each patient.

#### 1.5.2.1 SNPs in ER

Associations with hormone–dependent disease such as breast cancer and osteoporosis have beens found in studies on genetic variations in ER[230-232]. The most studied SNPs in ERα associated with an osteoporosis phenotype are two intronic SNPs located in the ERα

promoter[139]. However, study results are inconsistent[224,233,234]. Other studies were able to identify associations of SNPs in ERα with BMD as well as fracture risk for women and men[235,236]. Several SNPs in ERβ have been associated with BMD and fracture risk, but differences in distribution among populations need to be considered to understand the functional significance of those variations[230,237].

Several SNPs in ERα have been suggested to be associated with breast cancer risk in different populations, but none of these could be confirmed[238-241].

An important field of study is how genetic variations in ER contribute to estrogen-dependent treatment for diseases like breast cancer or osteoporosis. Women treated with tamoxifen for breast cancer carrying an intronic ERβ variant had increased BMD gain and less bone turnover if compared to WT[242]. Therefore, this polymorphism might be a predictive marker for the response to tamoxifen in bone[242].

# 2 Hypothesis and Objectives

Current treatment or prevention of hormone responsive breast cancer includes mainly SERMs like Tamoxifen or Raloxifene. Despite their antiestrogenic effect in breast they exert agonistic effects in other estrogen target tissues including bone. Raloxifene is approved for the treatment and prevention of osteoporosis. Estrogens and SERMs can inhibit bone loss in women due to decreased estrogen levels seen after menopause. Estrogen and SERMs primarily act by regulating gene transcription via estrogen receptors (ER). Further, ERα is regulated through its interaction with coactivators which can enhance its activity. Steroid Receptor Coactivator -1 (SRC-1) plays an important role in mediating the relative agonist/antagonist activities of the SERM tamoxifen, in the breast, endometrium and in the bone. Studies in mice lacking SRC-1 have revealed increased bone turnover and osteopenia, similar to the effects of estrogen deficiency. These changes are refractory to the administration of exogenous estrogen. Characterization of genetic variation in SRC-1 will lead to a better understanding of how the genetic background influences the development of breast cancer and osteoporosis and the efficiency of treatments. One nonsynonymous SNP exists in SRC-1 at amino acid position 1272 resulting in an amino acid change from proline to serine (P1272S). An exchange of proline to serine can confer an essential change in SRC-1 protein function. Furthermore, this genetic alteration is located in the AD2 of SRC-1 that is known to play an important role in regulating transcriptional activity.

Therefore, the underlying hypothesis is that SRC-1 P1272S alters ERα's transcriptional activity *in vitro* and *in vivo*, and hence hormone response in breast cancer patients in estrogen sensitive tissues such as bone and breast.

The research objectives addressed the following tasks:

1. To test alterations of SRC-1 P1272S coactivator activity compared to SRC-1 WT utilizing *in vitro* assays.
2. To study associations of SRC-1 P1272S with clinical phenotypes.
3. To test the role of SRC-1 and SRC-1 P1272S on bone maintenance using skeletal cell culture systems.
4. To determine the mechanism by which SRC-1 P1272S's coactivator activity is altered compared to SRC-1 WT.

# 3 Results

## 3.1 Sequencing SRC-1 coding regions

The completion of the International HapMap Project allows for identification of a continuously growing number of associations of genetic variations with common diseases and responses to drugs. SNPs in various genes have already been shown to contribute to a change in risk, outcome, or treatment efficiency for several diseases[184,206,207].

SRC-1 is an important NR coactivator with a pivotal role for the pathology of osteoporosis[69,243] and breast cancer[65,66]. Prior to these studies nothing was known about how SNPs in SRC-1 contribute to altered outcome in those diseases or endocrine treatment efficiency. This indicated it is imperative to study genetic alterations in SRC-1.

All validated SRC-1 coding region SNPs within the dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/index.html) were extracted (Jay Wang M.D., Ryan Hartmaier at BCM, Houston, USA). Of all the variants identified, rs1804645 represented the only non-synonymous SNP. To confirm the finding published within the dbSNP database, to gain additional population-specific information on rs1804645, and to identify potentially novel SNPs in SRC-1 direct sequencing of all SRC-1 coding exons in 48 Caucasian (CA) and 48 African-American (AA) apparently normal individuals (Coriell Institute, NJ) was carried out. From this effort a total of six variants were identified (Table 1).

Five of the SNPs found in the Coriell sequencing were present in dbSNP, and one was novel. Of all the variants identified during sequencing, rs1804645 represented the only non-synonymous SNP. This SNP changes a proline to serine at amino acid position 1272 in exon 18; it will be referred to as P1272S (Figure 9).

| rs# | Amino Acid Position | Amino Acid Change | Population | Samples (indiv) | MAF |
|---|---|---|---|---|---|
| rs1804645 | 1272 | P/S | CEPH | 92 | |
| | | | AGI_ASP population | 37 | 0.014 |
| | | | **Coriell resequencing - AA** | 48 | 0.010 |
| | | | **Coriell resequencing - CA** | 48 | 0.031 |
| rs11125763 | 1267 | L/L | AGI_ASP population | 37 | 0.189 |
| | | | HapMap-CEU | 59 | 0.110 |
| | | | HapMap-HCB | 45 | 0.000 |
| | | | HapMap-JPT | 45 | 0.011 |
| | | | HapMap-YRI | 60 | 0.500 |
| | | | **Coriell resequencing - AA** | 48 | 0.292 |
| | | | **Coriell resequencing - CA** | 48 | 0.073 |
| rs13430401 | 1068 | L/L | HapMap-CEU | 60 | 0.000 |
| | | | HapMap-HCB | 45 | 0.000 |
| | | | HapMap-JPT | 45 | 0.000 |
| | | | HapMap-YRI | 60 | 0.000 |
| | | | **Coriell resequencing - AA** | 48 | 0.031 |
| | | | **Coriell resequencing - CA** | 48 | 0.000 |
| rs41281515 | 641 | A/A | No Frequency Data in dbSNP | | |
| | | | **Coriell resequencing - AA** | 47 | 0.000 |
| | | | **Coriell resequencing - CA** | 48 | 0.010 |
| No rs# | 177 | L/L | Not found in dbSNP | | |
| | | | **Coriell resequencing - AA** | 48 | 0.010 |
| | | | **Coriell resequencing - CA** | 47 | 0.000 |
| rs11125744 | 154 | T/T | AFD_EUR_PANEL | 24 | 0.104 |
| | | | AFD_AFR_PANEL | 23 | 0.391 |
| | | | AFD_CHN_PANEL | 24 | 0.000 |
| | | | AGI_ASP population | 38 | 0.276 |
| | | | HapMap-CEU | 60 | 0.100 |
| | | | HapMap-HCB | 45 | 0.000 |
| | | | HapMap-JPT | 44 | 0.012 |
| | | | HapMap-YRI | 60 | 0.567 |
| | | | **Coriell resequencing - AA** | 48 | 0.417 |
| | | | **Coriell resequencing - CA** | 47 | 0.128 |

**Table 1: SNPs in SRC-1 coding regions.**

**SNPs were identified through dbSNP (**http://www.ncbi.nlm.nih.gov/projects/SNP/**) and by full exon sequencing of Coriell samples. Our re-sequencing is listed as "Coriell re-sequencing" with AA representing African Americans and CA representing Caucasian Americans. (AGI_ASP population is a mixture of African American and Caucasian samples. CEPH, HapMap-CEU, and AFD_EUR_PANEL represent populations of European descent. HapMap-HCB and AFD_CHN_PANEL populations are of Chinese descent. HapMap-YRI and AFD_AFR_PANEL are populations of African descent. HapMap-JPT is a population of Japanese descent. MAF=minor allele frequency.**

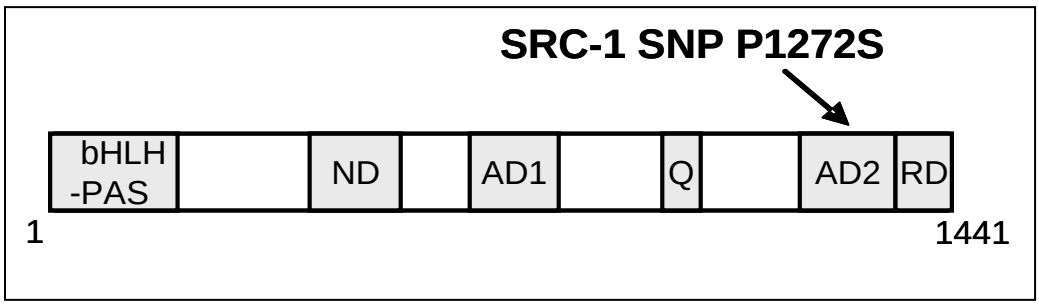



**Figure 9: Schematic presentation of functional SRC-1 domains, and position of SNP P1272S.**

**The non-synonymous SNP P1272S is located in the AD2 of SRC-1. bHLH-PAS= basic helix-loop-helix-Per-Arnt-Sim domain, AD = activation domain, NR = nuclear receptor interaction domain, Q = Glutamine rich region, RD = repression domain.**

## 3.2 SRC-1 P1272S shows decreased nuclear receptor co-activation

SRC-1 P1272S resides in the AD2 of SRC-1 (Figure 9), which is known to be critical for its coactivation function[244].

Using Polyphen (http://genetics.bwh.harvard.edu/pph/) in-situ analysis of this SNP predicted SRC-1 P1272S to be probably damaging (Jay Wang M.D., BCM, Houston, USA). Polyphen is a tool which predicts possible impact of an amino acid substitution on the structure and function of a human protein using physical and comparative considerations. Results range from benign to possibly damaging to probably damaging.

For further in vitro studies an SRC-1 expression plasmid, containing the change P1272S, was constructed by site directed mutagenesis.

### 3.2.1 SRC-1 P1272S decreases ERα coactivity

It was first tested whether the SRC-1 P1272S variant demonstrated altered coactivation of ERα in different cell lines. Transient estrogen-responsive luciferase reporter assays were conducted in breast cancer cells (MCF-7, T47D), endometrial carcinoma cells (Ishikawa), cervical cancer cells (Hela), liver carcinoma cells (HepG2) and human embryonic kidney cells (HEK293). The ER-negative cell lines HepG2, Hela, HEK293 were additionally cotransfected with a pCDNA3.1-HA-ERα construct. Cell lines were selected due to their known ER-, SRC-1- expression level or high transfection efficiency.

As shown in Figure 10 the transient transfection of SRC-1 WT resulted in an expected increase in ERα coactivation after estrogen treatment. Transfection of SRC-1 P1272S however showed a significant diminished coactivation of ERα in the presence of the variant in various cell lines compared to SRC-1 WT.

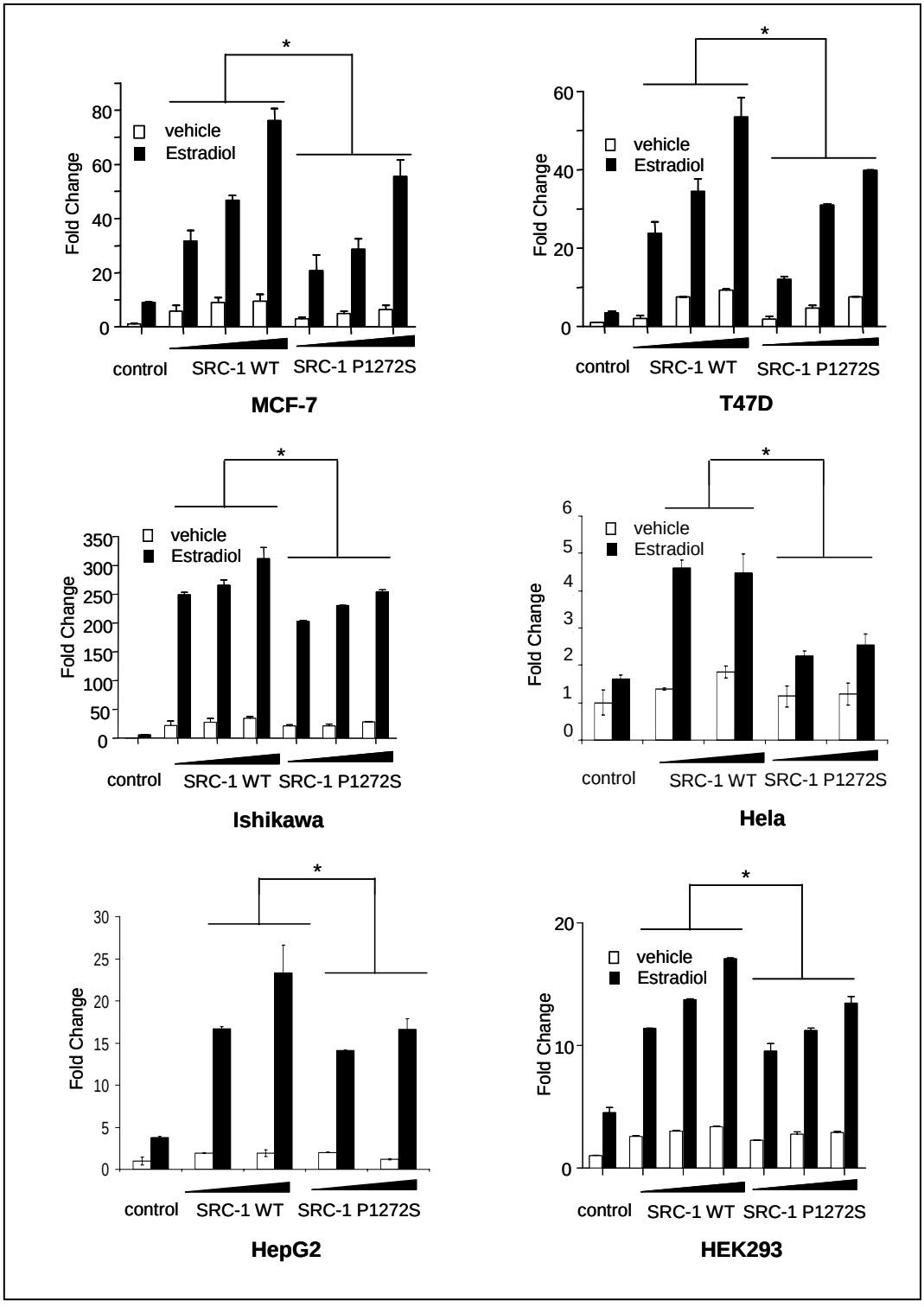

*
vehicle
Estradiol
Fold Change
control
SRC-1 WT
SRC-1 P1272S
MCF-7
T47D
Ishikawa
Hela
HepG2
HEK293

**Figure 10: Estrogen receptor activity is partially attenuated in the presence of SRC-1P1272S.**

**Cells were transiently transfected with 100ng ERE-TK-Luc reporter construct, and increasing amounts (100ng, 200ng, or 500ng) of expression constructs for pSG5-SRC-1 WT or pSG5-SRC-1 P1272S. The ER-negative cell lines HepG2, Hela, HEK293 were additionally cotransfected with 25ng of a pCDNA3.1-HA-ERα construct. Cells were treated with vehicle or estradiol ($10^{-8}$M) for 24h. Relative Luciferase Units were determined and normalized against total protein. The data is presented as fold over control, relative to untreated vehicle. The data shown are representative of at least 3 independent experiments for MCF7, T47D, Ishikawa, Hela, HepG2, and HEK293 cells respectively. Error bars represent standard deviation. p –value was calculated using two-way ANOVA test (*, $p< 0.05$).**

### 3.2.2 SRC-1 P1272S decreases Progesterone Receptor coactivity

Due to SRC-1's capability to coactivate a broad range of NRs, experiments to investigate if the observed decrease in coactivity is confined to ERα were performed. To study the effect of SRC-1 on PR coactivation the uterine cell line Ishikawa was used, since PR and SRC-1 are highly expressed and colocalize in the uterus[245]. When SRC-1 WT and P1272S coactivation was studied using progesterone-responsive luciferase reporter assays in Ishikawa cells, similar results were obtained. In the presence of progesterone SRC-1 WT induced PR activity. In the presence of SRC-1 P1272S progesterone dependent PR coactivation was diminished (Figure 11). These results suggest that the decreased coactivity in the presence of SRC-1 P1272S is not only decreasing ERα coactivity, but also reduces PR transcriptional activity.

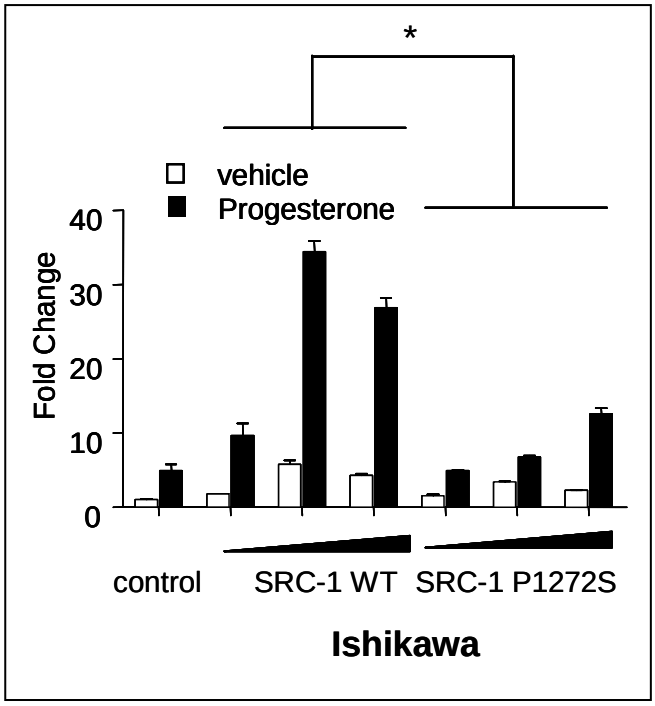



**Figure 11: Progesterone receptor activity is partially attenuated in the presence of SRC-1 P1272S.**

**Cells were transiently transfected with 100ng PRE-TATA-LUC reporter construct, and increasing amounts (100ng, 200ng, or 500ng) of expression constructs for pSG5-SRC-1 WT or pSG5-SRC-1 P1272S. Cells were treated with vehicle or progesterone ($10^{-8}$M). Relative Luciferase Units were determined and normalized against total protein. The data is presented as fold over control relative to untreated vehicle (shown is a representative result from 2 independent experiments). Error bars represent standard deviation. p –value was calculated using two-way ANOVA test (*, $p< 0.05$).**

## 3.3 Genotyping for SRC-1 P1272S

To determine whether these *in vitro* findings may play a role in breast cancer etiology and response to endocrine therapy, human cell lines and clinical samples were genotyped for SRC-1 P1272S. These studies were conducted within the COBRA network.

### 3.3.1 Genotyping cell lines for SRC-1 P1272S

In collaboration with the University of Michigan, 28 cancer cell lines, including 21 breast cancer cell lines were genotyped for SRC-1 P1272S. This would give insights whether SRC-1 P1272S could serve as a risk factor for developing estrogen-dependent breast cancer.

SRC-1 P1272S did not occur in any of the screened breast cancer cell lines, suggesting that the SNP might exert a protective effect on developing hormone-dependent breast cancer. However, this conclusion is subject for reconsideration, since only cancer cell lines were genotyped. The comparison to normal cell lines remains elusive.

Out of 28 cancer cell lines, only the colon cancer cell line Caco-2 was identified to be heterozygous for the SNP (Table 2).

| Cell line | Cell type | SRC1 genotype |
|---|---|---|
| A-431 | Skin | C/C |
| BT-20 | Breast | C/C |
| BT-474 | Breast | C/C |
| BT-549 | Breast | C/C |
| ***Caco-2*** | ***Colon*** | ***C/T*** |
| HBL-100 | Breast | C/C |
| Hep3B | Liver | C/C |
| HepG2 | Liver | C/C |
| LCC-6 | Breast | C/C |
| LS-174T | Colon | C/C |
| LY2 | Breast | C/C |
| MCF-7 p180 | Breast | C/C |
| MCF-7 p22 | Breast | C/C |
| MDA-MB-134 | Breast | C/C |

| Cell line | Cell type | SRC1 genotype |
|---|---|---|
| MDA-MB-231 | Breast | C/C |
| MDA-MB-361 | Breast | C/C |
| MDA-MB-435 | Skin | C/C |
| MDA-MB-453 | Breast | C/C |
| MDA-MB-468 | Breast | C/C |
| SKBR3 | Breast | C/C |
| SKOV-3 | Ovary | C/C |
| SUM 159 | Breast | C/C |
| SUM 229 | Breast | C/C |
| SUM 52 PE | Breast | C/C |
| T47D | Breast | C/C |
| ZR-75-1 | Breast | C/C |
| ZR-75-30 | Breast | C/C |
| ZR-75-B | Breast | C/C |

**Table 2: Genotpying cancer cell lines for SRC-1 P1272S.**

**28 cancer cell lines were genotyped for SRC-1 P1272S. Cell lines, cell origin, and genotype of SRC-1 P1272S are given in the table. SRC-1 genotype is defined with C/C for SRC-1 WT and with C/T for SRC-1 P1272S (3814 C>T) (C- Cytosine; T- Thymine).**

### 3.3.2 SRC-1 P1272S is not associated with breast cancer risk

To further test if carrying SRC-1 P1272S results in a decreased risk of developing breast cancer, a case control study for association with breast cancer risk was performed in collaboration with the Deutschem Krebsforschungszentrum (DKFZ) in Heidelberg. For this study 1509 healthy controls and 1218 familial breast cancer patients, which were selected to be negative for BRCA1/2 mutations, were genotyped for SRC-1 P1272S. Among the controls, 1432 WT and 77 people were identified to be heterozygous for the SNP. Among the cases 1147 patients were WT, 69 heterozygous and 2 homozygous for SRC-1 P1272S. The variant did not show an association with breast cancer in this particular study (table 3).

| | Genotype | Cases | Controls | OR | 95% CI | P |
|---|---|---|---|---|---|---|
| **SRC-1 P1272S (3814 C>T)** | CC (%) | 1147 (94.2) | 1432 (94.9) | 1 | | |
| | CT (%) | 69 (5.6) | 77 (5.1) | 1.11 | 0.80-1.56 | >0.05 |
| | TT (%) | 2 (0.2) | 0 (0.0) | 6.24 | 0.29-130.14 | >0.05 |
| | [CT + TT] <-> [CC] | | | 1.15 | 0.82-1.60 | >0.05 |

**Table 3: Case-Control study for association with breast cancer risk.**

**1218 familial breast cancer patients and 1509 healthy controls (negative for BRCA1/2 mutations) were genotyped for SRC-1 P1272S. Genotype frequencies of SRC-1 P1272S, odd ratios (OR) with 95% confidence intervals (CI) and P-values are given in the table.**

### 3.3.3 Loss of BMD in tamoxifen treated women with SRC-1 P1272S

To determine whether the observed decreased coactivity in the presence of SRC-1 P1272S plays a role in response to endocrine breast cancer therapy, we genotyped for the variant in the COBRA tamoxifen trial. This trial enrolled pre- and postmenopausal women between 18 and 60 years of age, who took the SERM Tamoxifen for breast cancer prevention or treatment. DNA was collected as part of a prospective clinical trial which was designed to associate genetic variants with well-curated phenotypic outcomes, including BMD, in response to Tamoxifen[246] (http://www.pharmgkb.org/contributors/pgrn/cobra_profile.jsp). This study was conducted within the COBRA network.

For 204 women, data for hip and lumbar BMD, measured by DXA scanning before the start of Tamoxifen treatment, and 12 months later, were available. A subset of those women (n=93) had also received chemotherapy for their breast cancer treatment. Chemotherapy frequently induces early menopause in premenopausal women, and can cause an up to 14% lower BMD[247]. Therefore, patients who received additionally chemotherapy were excluded from the analysis.

We asked the question whether women carrying the SRC-1 P1272S variant, and who received Tamoxifen, had lower BMD. Genotyping revealed that there were 4 heterozygous SNP carriers among the 111 women included in the study. For 5 additional patients lumbar BMD measurements, but no hip BMD information were available (Hip BMD n=106; lumbar BMD n=111).

There was no significant difference in baseline BMD between WT and P1272S carriers. A decrease of 0.6% for hip and of 1.3% for lumbar BMD was observed after 12 months tamoxifen treatment in women genotyped for SRC-1 WT. However, following 12 months of treatment with Tamoxifen, women carrying the SRC-1 P1272S allele showed a decrease in BMD compared to women carrying the WT allele (Figure 12). Hip BMD decreased by 4.3% for SNP carriers (n=4) versus 0.6% in the WT group (n=102), however due to the low frequency of the SNP this association did not reach statistical significance.

Similar results were obtained for lumbar BMD, where the difference reached statistical significance; in SRC-1 P1272S carriers (n=4) lumbar BMD was decreased by 6.4%, compared to 1.3% decrease in women without the SNP (n=107) ($p<0.05$). This data suggests that women carrying SRC-1 P1272S might be at increased risk for bone mineral loss.

Our results further imply that carrying SRC-1 P1272S might reflect a loss of tamoxifen's agonist activity on bone formation.

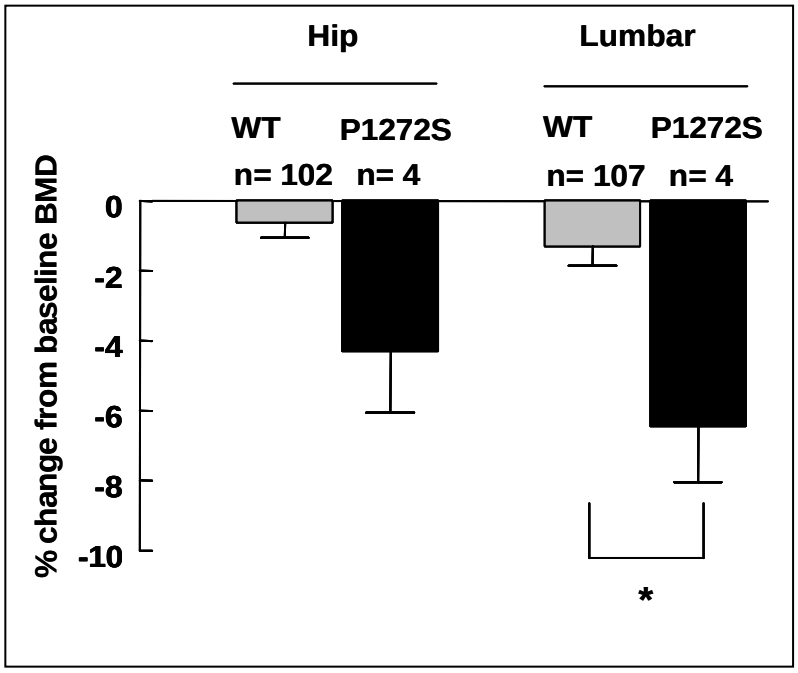



**Figure 12: Decreased BMD in SRC-1 P1272S carriers receiving tamoxifen.**

**Hip and lumbar BMD was measured by DXA at baseline and after 12 months of tamoxifen treatment. Data for hip and lumbar BMD was available for 106 and 111 patients, respectively. Patients carrying the SRC-1 P1272S allele showed a decrease in lumbar BMD (n=4) of 6.4% ($p<0.05$) and hip BMD (n=4) of 4.3% ($p>0.05$). Error bars represent SEM (*, $p< 0.05$).**

## 3.4 SRC-1 is important for tamoxifen's agonistic activity *in vitro*

### 3.4.1 SRC-1 P1272S decreases tamoxifen agonistic activity *in vitro*

Despite its antiestrogenic effect in the breast, tamoxifen acts as an agonist in organs like bone and liver. Our data revealed that women carrying SRC-1 P1272S show decreased BMD after 12 months of tamoxifen treatment (figure 12). This suggests that in the presence of the SNP the agonistic properties of tamoxifen in the bone are decreased.

It was tested if SRC-1 P1272S decreases tamoxifen's agonistic activity by using *in vitro* Luciferase reporter assays. For this experiment a human hepatocellular liver carcinoma cell line (HepG2) was used. ERE-luciferase reporter assays in HepG2 cells showed that transient transfection of SRC-1 WT resulted in an expected increase in ERα coactivation after tamoxifen treatment. In the presence of the SNP tamoxifen's agonistic activity is decreased if compared to SRC-1 WT (figure 13).

These results suggest that the decrease in ERα coactivity caused by SRC-1 P1272S diminishes the ability of tamoxifen to act as an agonist in cell lines from certain tissues.

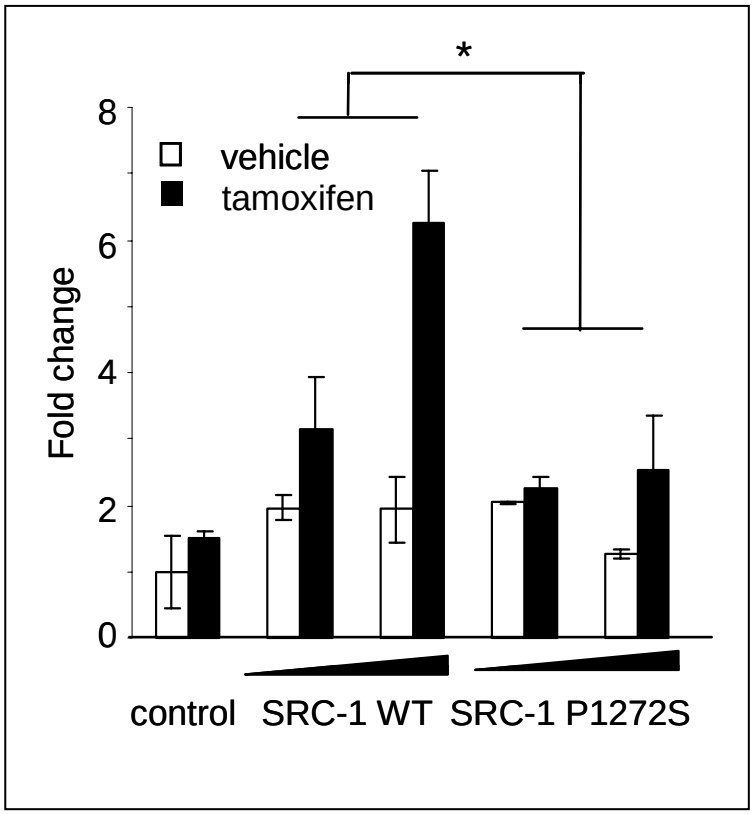



**Figure 13: Tamoxifen's agonist activity is lost in presence of SRC-1 P1272S.**

**HepG2 cells were transiently transfected with 100ng ERE-Tk-LUC reporter construct, 25ng of a pCDNA3.1-HA-ERα construct, and increasing amounts of DNA for pSG5-SRC-1 WT or pSG5-SRC-1 P1272S expression constructs. Cells were treated with vehicle (ethanol), or $10^{-6}$M tamoxifen. Relative Luciferase Units were determined and normalized against total protein. The data is presented as fold over control, relative to untreated vehicle (shown is a representative resulting from 2 independent experiments). Error bars represent standard deviation; p –value was calculated using a two-way ANOVA test (*, $p< 0.05$).**

### 3.4.2 Estrogen fails to induce normal osteoclast apoptosis in skeletal cell cultures from SRC-1 knock-out mice rescued with SRC-1 P1272S

The skeletal response to estrogen is impaired in female SRC-1 ko mice[243]. Estrogen induces apoptosis in osteoclasts[151].

Therefore, if the decreased ERα coactivation in the presence of SRC-1 P1272S influences the apoptotic response of osteoclasts to estrogen was tested.

Bone marrow cells from SRC-1 ko mice were differentiated into a mixed skeletal cell culture containing mainly pre-osteoclasts, and mature osteoclasts. Bone marrow cells from SRC-1 ko mice were isolated from tibia and femur. The cells were differentiated in the presence of macrophage colony stimulating factor (M-CSF) and receptor activator for nuclear factor κ B ligand (RANKL) as previously described[163,168]. TRAP staining was used as a marker for osteoclastic activity and showed that approximately 30% of bone marrow cells were successfully differentiated into TRAP-positive preosteoclasts and osteoclasts (figure 14A).

These cell cultures were transiently transfected with either WT SRC-1 or SRC-1 P1272S and treated with estrogen for 16 hours. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed to detect apoptotic cells.

As shown in figure 14B estrogen increased osteoclast apoptosis in the cells transfected with SRC-1 WT, as expected. However, this response was attenuated in the cultures transfected with SRC-1 P1272S as well as in untransfected cultures (figure 14B).

These data strongly suggest that SRC-1 P1272S attenuates the response of these cells to estrogen, leading to a decrease in osteoclast apoptosis and subsequent increased bone turnover, and therefore could serve as a model for the decreased BMD observed in women who carry the SRC-1 P1272S SNP.

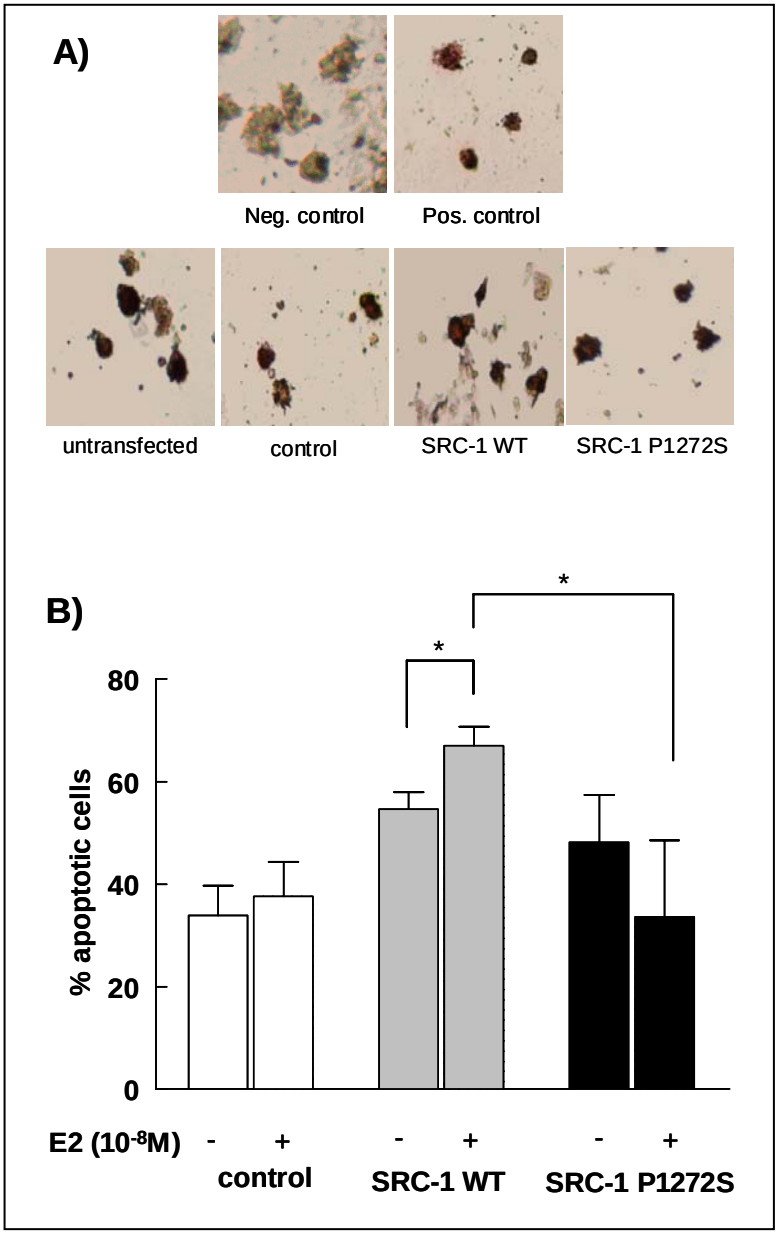



**Figure 14: Estrogen fails to induce apoptosis in the presence of SRC-1 P1272S.**

**A) TRAP staining for osteoclasts.**

**B) Cells were transfected with 200ng pSG5 (control), pSG5- SRC-1 WT, or pSG5- SRC-1 P1272S expression constructs and treated with either ethanol or estrogen ($10^{-8}$M) for 16 hours. Cells were then fixed and apoptosis was detected by determining the ratio of TUNEL-positive cells and DAPI-counterstained cells. Error bars represent SEM. Student's t-test was performed for pSG5- SRC-1 WT vehicle versus estradiol and pSG5- SRC-1 WT estradiol versus pSG5- SRC-1 P1272S estradiol (*, $p<0.05$). (Shown is a representative result from 2 independent experiments).**

### 3.4.3 Fas Ligand

Apoptosis of osteoclasts can be induced by a paracrine mechanism in which estrogen affects osteoclast survival through the upregulation of FasL in osteoblasts[168]. Krum et *al.* reported that estrogen induces FasL expression in osteoblastic cells (U2OS –Flag ERα) significantly within three hours[168].

The observed decreased apoptosis rate of osteoclasts in the presence of SRC-1 P1272S could be a result of decreased estrogen-induction of FasL compared to SRC-1 WT. Overexpression of SRC-1 WT would increase the induction of FasL after estrogen treatment and this response would be diminished in the presence of SRC-1 P1272S.

U2OS-Flag ERα cells were transfected with either pSG5 or pSG5- SRC-1 WT and treated with estrogen for three hours. However, a consistent estrogen dependent FasL induction was not observed in the performed experiments. The majority of performed experiments (n=7) show that overexpression of SRC-1 WT does not significantly increase the estrogen-dependent induction of FasL (figure 15).

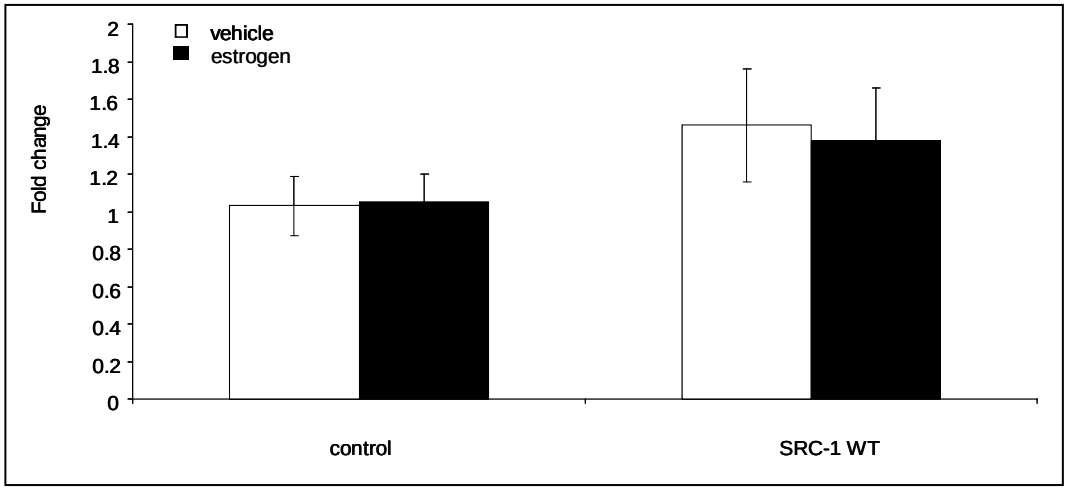



**Figure 15: Estrogen treatment does not result in an increase in FasL expression.**

**U2OS Flag-ERα cell were transfected with control vector or SRC-1 WT and treated with either vehicle or estrogen ($10^{-8}$M) for three hours. Total RNA was harvested and subjected to qRT PCR using human FasL and β-Actin specific primers to determine FasL expression. Data is expressed as FasL mRNA abundance relative to β-Actin levels. The data is presented as fold over control. Error bars represent SEM. Student's t-test was performed for vehicle versus estrogen in control and SRC-1 WT transfected cells, as well as for control estrogen versus SRC-1 WT estrogen.**

### 3.4.4 SRC-1 induces TIEG in a hormone dependent manner

Estrogen can directly regulate osteoblast proliferation and differentiation[248]. Estrogen stimulates the production of TGF-β by osteoblastic cells[249]. TGF-β slows the osteoclast activity and induces apoptosis of osteoclasts[250].

Overexpression of TIEG enhances TGF-β functions[251]. In osteoblasts TIEG is rapidly induced in response to estrogen by ERβ but not ERα[169]. Further, SRC-1 is essential for the estrogen induction of TIEG expression by ERβ[169].

That overexpression of SRC-1 WT can induce TIEG in reponse to estrogen in osteoblastic cell lines expressing ERβ needed to be confirmed. A pSG5- SRC-1 WT expression construct was transfected into U2OS-Flag ERβ cells and cells were treated with estrogen for one hour. As shown in figure 16 overexpression of SRC-1 resulted in a three-fold induction of TIEG mRNA after estrogen treatment compared to a control plasmid.

Since tamoxifen shows estrogenic activity in bone, it needed to be elucidated if tamoxifen induces TIEG similarly to estrogen. U2OS Flag-ERβ cells were treated over a timecourse of four hours with either estrogen or tamoxifen. As previously described for estrogen[169], our preliminary data shows that tamoxifen induces TIEG expression rapidly and transiently (figure 17).

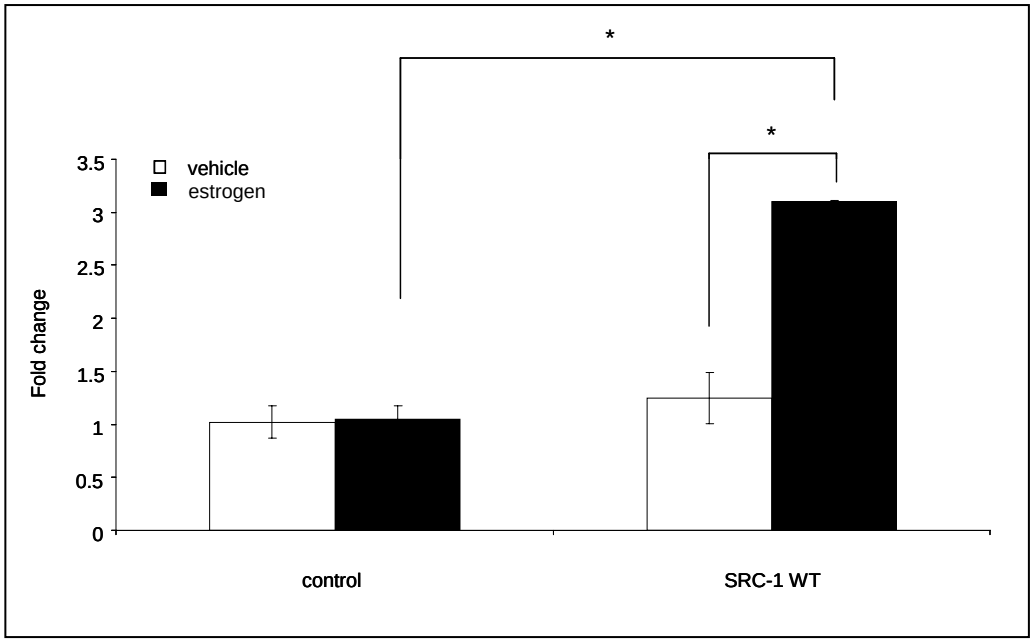



**Figure 16: SRC-1 can induce TIEG expression in response to estrogen.**

**U2OS Flag-ERβ cells were transfected with pSG5 (control) or pSG5- SRC-1 WT and treated with either vehicle (ethanol) or estrogen ($10^{-8}$M) for one hour. Total RNA was harvested and subjected to qRT PCR using human TIEG and β-Actin specific primers to determine TIEG expression. Data is expressed as TIEG mRNA abundance relative to β-Actin levels. The data is presented as fold over control. Error bars represent SEM. (Shown is a representative result from 3 independent experiments). Student's t-test was performed for control estrogen versus SRC-1 WT estrogen and for SRC-WT vehicle versus SRC-1 WT estrogen (*, $p<0.05$).**

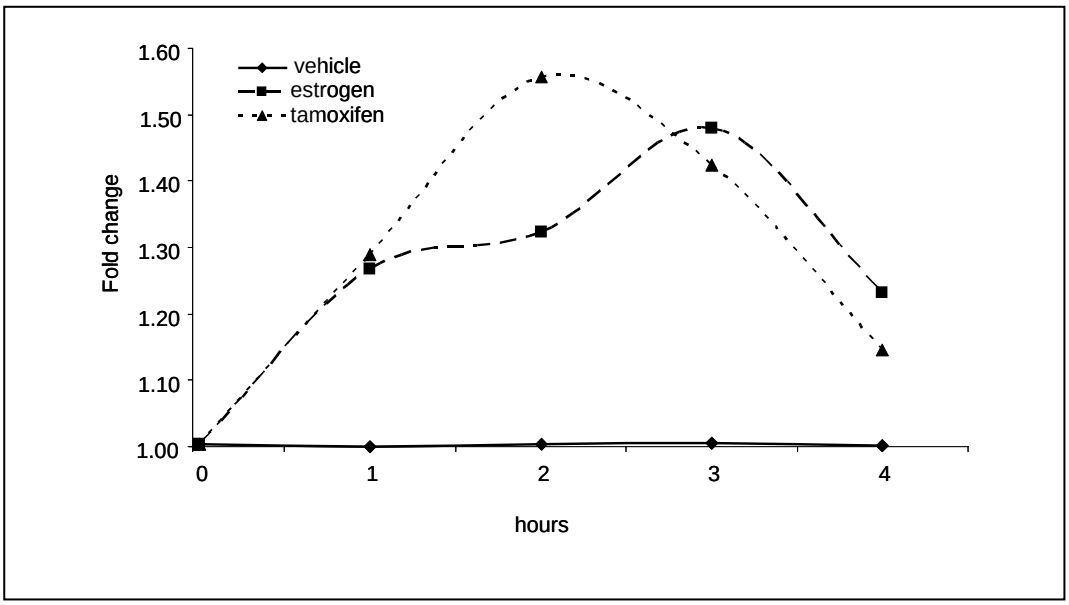



**Figure 17: Hormone treatment induces TIEG expression rapidly but transiently.**

**U2OS Flag-ERβ cells were treated with either vehicle (ethanol), estrogen ($10^{-8}$M), or tamoxifen ($10^{-6}$M) over a time course of four hours. Total RNA was harvested and subjected to qRT PCR using human TIEG and β-Actin specific primers. Data is expressed as TIEG mRNA abundance relative to β-Actin levels. The data is presented as fold over vehicle (n=1).**

#### 3.4.4.1 TIEG expression is decreased in the presence of SRC-1 P1272S

We wanted to test if SRC-1 P1272S changes the induction of TIEG compared to SRC-1 WT. U2OS Flag ERβ cells were transfected with pSG5 (control), pSG5- SRC-1 WT, or pSG5- SRC-1 P1272S. Cells were treated with vehicle (ethanol), estrogen, or tamoxifen for three hours. Transfection of SRC-1 WT resulted in a significant increase of TIEG mRNA levels after estrogen as well as tamoxifen treatment. However, SRC-1 P1272S transfection failed to upregulate TIEG expression for each treatment group (figure 18A). These results need to be considered preliminary until completely established.

To confirm that SRC-1 induces TIEG in response to hormone by ERβ but not ERα[169], the TIEG mRNA expression in U2OS Flag ERα cells was also determined after transfecting as described a control plasmid, SRC-1 WT, or SRC-1 P1272S. Cells were treated with vehicle, estrogen, or tamoxifen for three hours. This preliminary experiment showed that there was no change in TIEG expression when all three transfected plasmids were compared (figure 18B). Further, overexpression of SRC-1 WT did not result in increased TIEG expression in response to hormone. This would support that the SRC-1 dependent induction of TIEG is ERβ specific[169].

These data show that SRC-1 is an important cofactor for ERβ in upregulating TIEG. This suggests that the decreased TIEG expression in the presence of the SRC-1 P1272S might result in decreased osteoblastic TGF-β production and subsequently in decreased osteoclast apoptosis (figure 14B) and needs to be further evaluated.

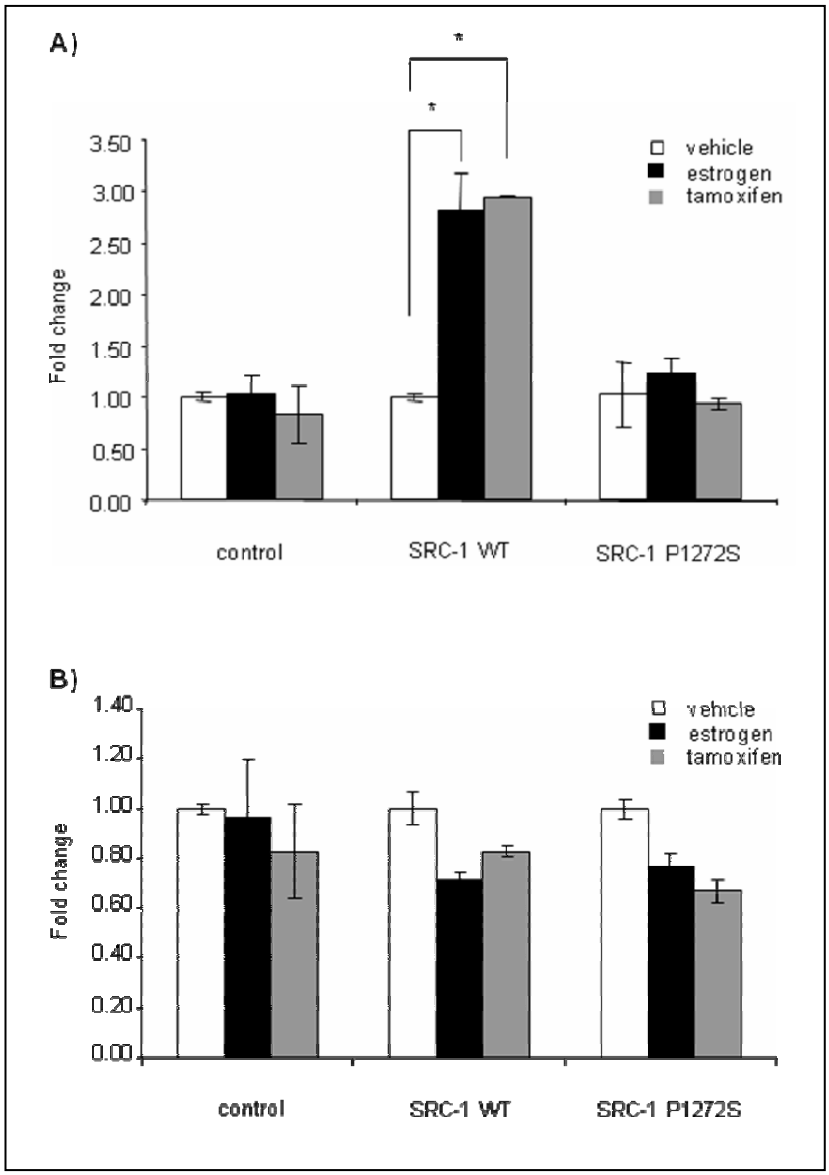

A)
*
*
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
Fold change
vehicle
estrogen
tamoxifen
control
SRC-1 WT
SRC-1 P1272S
B)
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Fold change
vehicle
estrogen
tamoxifen
control
SRC-1 WT
SRC-1 P1272S

**Figure 18: TIEG expression is ERβ-dependent decreased in the presence of SRC-1 P1272S.**

**Cells were transfected with pSG5 (control), pSG5-SRC-1 WT, or pSG5-SRC-1 P1272S and treated with vehicle, estrogen ($10^{-8}$M), or tamoxifen ($10^{-6}$M) for three hours. Total RNA was harvested and subjected to qRT PCR using human TIEG and β-Actin specific primers. Data is expressed as TIEG mRNA abundance relative to β-Actin levels. Error bars represent SEM. The data is presented as fold over control relative to vehicle.**

**(A) U2OS Flag ERβ cells (n=3). Student's t-test was performed for vehicle versus estrogen for pSG5, WT, and P1272S as well as vehicle versus tamoxifen for pSG5, WT, and P1272S (*, $p< 0.05$).**

**(B) U2OS Flag ERα cells (n=1). Student's t-test was performed as described for (A) with reaching no significance for all comparisons.**

### 3.4.5 The role of SRC-1 on osteoblast proliferation and differentiation

Due to the decrease of TIEG expression in the presence of the SRC-1 P1272S, we wanted to see if osteoblasts derived from SRC-1 ko mice also show a decrease in TIEG expression. This could demonstrate that SRC-1 is needed to activate TIEG in a hormone-dependent manner and might subsequently explain the role of SRC-1 in maintaining BMD.

Osteoblasts from SRC-1 WT, SRC-1 heterozygous (het) and SRC-1 ko mice were isolated from calvaria of five-day old mice. To release osteoblasts the calvaria were digested with collagenase P.

SRC-1 mRNA expression was confirmed after genotyping. Osteoblasts from SRC-1 WT, SRC-1 het, and SRC-1 ko mice express ERα and ERβ (figureS1A and B).

Cells were treated with vehicle (ethanol), estrogen, or tamoxifen for four hours. QRT-PCR was performed for TIEG in SRC-1 WT, SRC-1 het, and SRC-1 ko derived osteoblasts. Estrogen as well as tamoxifen increased TIEG mRNA level in osteoblasts derived from SRC-1 WT mice more than two-fold. SRC-1 het osteoblasts showed an increase in TIEG levels but to a lesser extent compared to SRC-1 WT. Importantly, SRC-1 ko osteoblasts did not show increased TIEG mRNA level in response to hormone (figure 19).

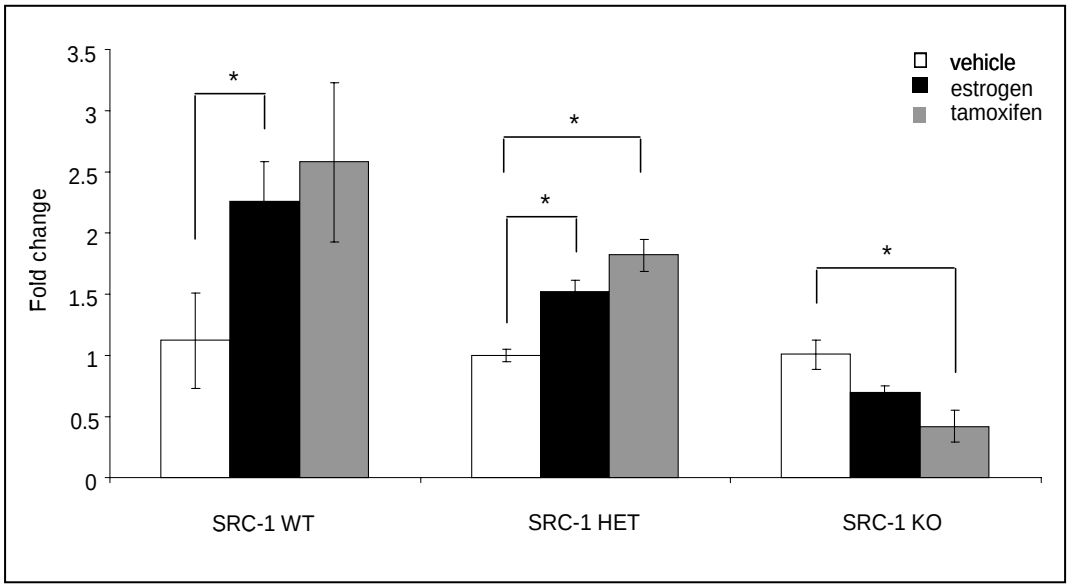



**Figure 19: SRC-1 is needed for hormone dependent induction of TIEG.**

**Total RNA was harvested and subjected to qRT PCR using mouse TIEG and β-Actin specific primers. Data is expressed as TIEG mRNA abundance relative to β-Actin levels. The data is presented as fold over vehicle (n=1). Error bars represent SEM. Student's t-test was performed for vehicle versus estrogen for SRC-1 WT, SRC-1 het, and SRC-1 ko as well as vehicle versus tamoxifen for SRC-1 WT, SRC-1 het, and SRC-1 ko (*, $p < 0.05$).**

In addition to inducing osteoclast apoptosis TGF-β regulates cell proliferation and differentiation of osteoblasts[252,253].

Based on the observations that in the absence of SRC-1 TIEG expression levels are decreased, it was of interest to determine the role of SRC-1 in osteoblast proliferation and differentiation.

To test if SRC-1 affects osteoblast proliferation a MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay was performed. The MTS assay is a colorimetric method for measuring the activity of enzymes that reduce MTS + phenazine methosulfate (PMS) to formazan. The quantity of formazan product as measured by absorbance at 490nm is directly proportional to the number of living cells in culture.

SRC-1 WT, SRC-1 het, and SRC-1 ko osteoblasts were treated with vehicle (ethanol), estrogen, or tamoxifen over a timecourse of 48 hours. Osteoblasts derived from SRC-1 ko mice show less cell proliferation for all treatment groups at all timepoints compared to osteoblasts derived from SRC-1 WT or SRC-1 het mice (figure 20). At 16 hours of hormone treatment estrogen as well as tamoxifen increased cell proliferation in SRC-1 WT and SRC-1 het osteoblasts. Importantly, estrogen or tamoxifen were not able to induce cell proliferation of osteoblasts derived from SRC-1 ko mice at all timepoints (figure 20).

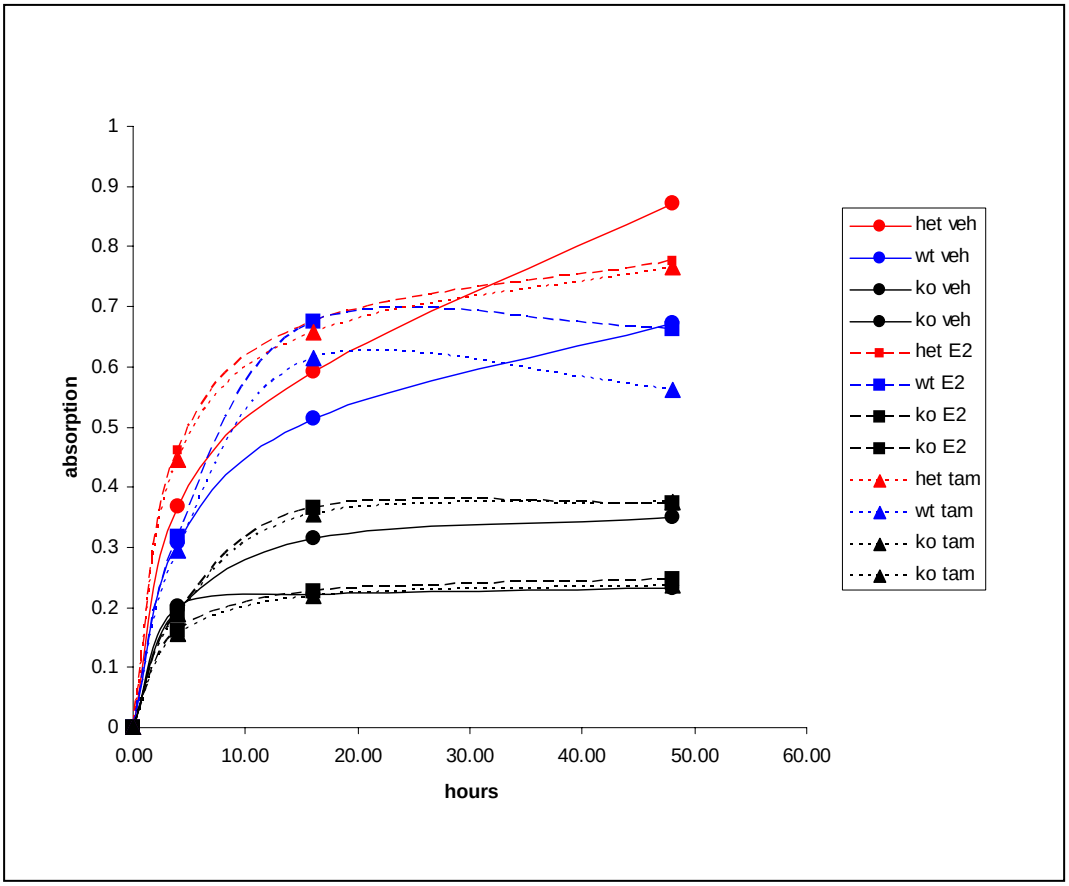



**Figure 20: SRC-1 ko osteoblasts show diminished cell proliferation (n=1).**

**Osteoblasts derived from SRC-1 WT (blue), SRC-1 het (red), or SRC-1 ko (black) were treated with vehicle (●), estrogen (■), or tamoxifen (▲). Absorption at 490nm was determined at at 4 hours, 16 hours, and 48 hours.**

TIEG ko mice show decreased expression of osteoblast differentiation markers like alkaline phosphatase (ALP)[174].

To test if osteoblast differentiation is altered in the absence of SRC-1, qRT PCR for ALP was performed in the osteoblasts derived from SRC-1 WT, SRC-1 het, and SRC-1 ko mice. Osteoblasts were treated with vehicle, estrogen, or tamoxifen for four hours.

Tamoxifen induced ALP significantly in osteoblasts derived from SRC-1 WT if compared to vehicle. SRC-1 het and SRC-1 ko osteoblasts did not show a significant increase in ALP mRNA expression levels after tamoxifen treatment. Estrogen treatment at the same time showed a slight but not significant increase in ALP mRNA levels for SRC-1 WT osteoblasts that was not seen with SRC-1 het or ko osteoblasts (figure 21). Although further evaluation is necessary this suggests that SRC-1 might be needed to induce hormone-dependent ALP expression in osteoblasts and that SRC-1 may play an important role for osteoblast differentiation.

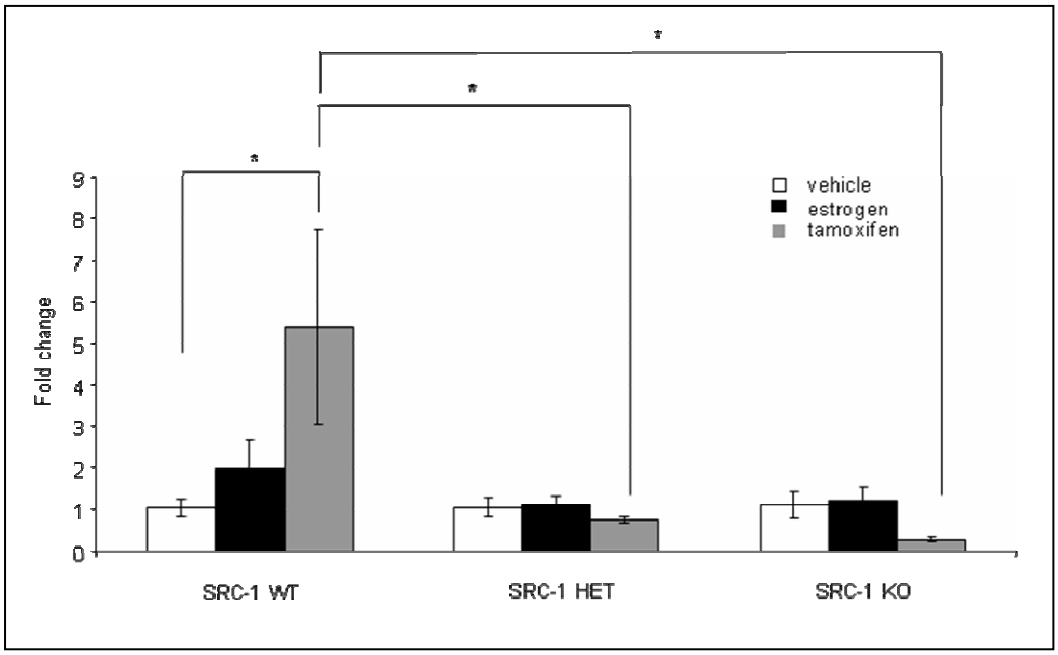



**Figure 21: SRC-1 is needed for hormone dependent induction of Alkaline Phosphatase (ALP).**

**Total RNA was harvested and subjected to qRT PCR using mouse ALP and β-Actin specific primers. Data is expressed as ALP mRNA abundance relative to β-Actin levels. The data is presented as fold over vehicle (n=1). Error bars represent SEM. Student's t-test was performed in the estrogen group for SRC-1 WT versus SRC-1 het and SRC-1 ko as well as for tamoxifen SRC-1 WT versus SRC-1 het and ko; for SRC-1 WT vehicle versus estrogen and SRC-1 WT vehicle versus tamoxifen (*, $p < 0.05$).**

## 3.5 SRC-1 P1272S leads to changes in phosphorylation sites that impact degradation

### 3.5.1 SRC-1 P1272S protein degrades faster than SRC-1 WT

The activity of SRC-1 is, at least in part, regulated via its degradation through the proteasome pathway[254]. It was therefore tested if SRC-1 P1272S resulted in altered degradation of the protein if compared to SRC-1 WT. To address protein degradation, experiments using cycloheximide as translational inhibitor as well as pulse chase experiments were performed.

Cycloheximide inhibits protein biosynthesis by blocking translational elongation. It is generally used to study degradation and subsequently half-life of a protein as protein *de novo* synthesis is inhibited in the presence of cycloheximide.

Hela cells were transiently transfected with flag-tagged constructs for either pSG5-SRC-1 P1272S or pSG5-SRC-1 WT. Throughout the experiment cells were cultured in full serum medium, which contains estrogen at physiological levels. Knowing that the half-life of the SRC family member SRC-3 is 2-3 hours[42], the transfected cells were treated with cycloheximide over a time course of four hours. Transfected flag- tagged SRC-1 protein was detected by western blot using an anti-flag-antibody. As shown in figure 22A SRC-1 P1272S degrades faster than SRC-1 WT. Quantification of the westernblot reveals that SRC-1 P1272S protein shows a shorter half-life compared to SRC-1 WT.

To confirm differences in protein degradation between SRC-1 WT and SRC-1 P1272S, pulse chase experiments were performed. In this experiment cells are incubated with radiolabeled [$^{35}$S] methionine for a short period (pulse labeling) to radiolabel newly synthesized SRC-1 protein. The pulse period is followed by a chase period in which cells are further incubated with excess amount of the unlabeled counterpart of the precursor used for labeling. After isolation from other cellular proteins by immunoprecipitation the radiolabeled SRC-1 is analyzed by electrophoresis and detected by autoradiography.

Specifically, HEK293 cells were transfected with either pSG5-SRC-1 WT or pSG5-SRC-1 P1272S and subsequently treated with [$^{35}$S] methionine. Cells were chased in full serum medium over a time course of 16 hours. As shown in figure 22B SRC-1 P1272S degraded faster compared to SRC-1 WT, confirming results seen in cycloheximide experiments.

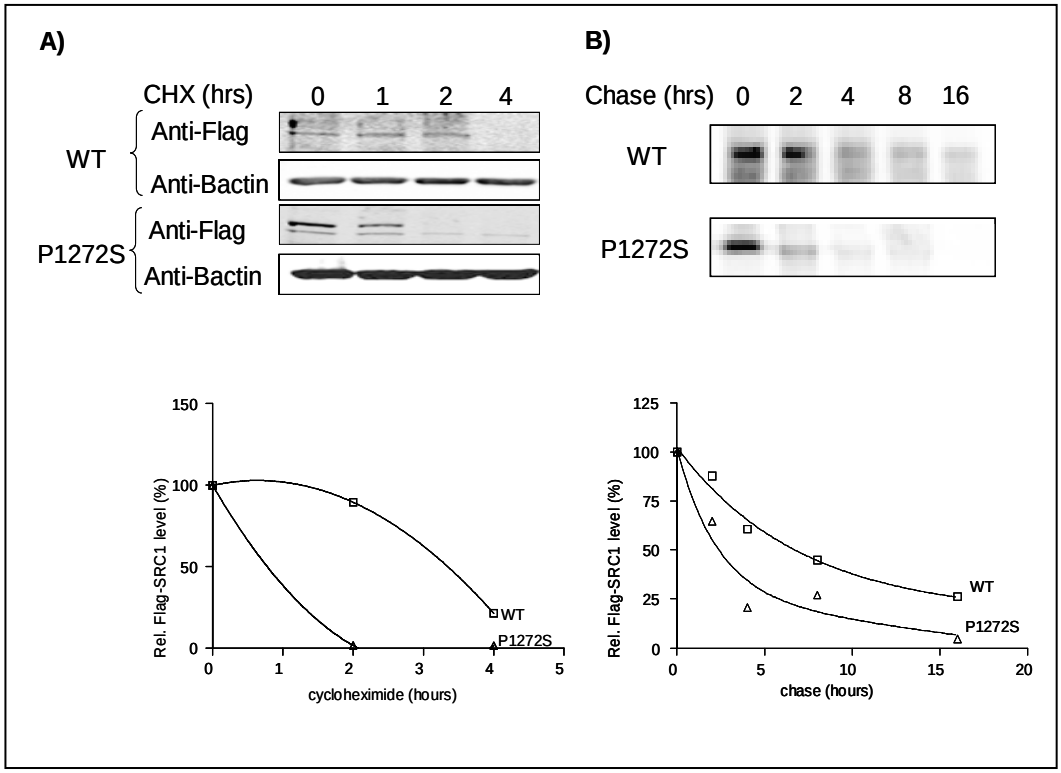



**Figure 22: Differences in turnover rates between SRC-1 WT and SRC-1 P1272S.**

**A) Half-life of Flag-SRC-1 P1272S compared to WT Flag-SRC-1 in the presence of cycloheximide. (The data shown is representative of at least 3 independent experiments.)**

**B) Pulse-Chase of Flag-SRC-1 P1272S compared to Flag-SRC-1 WT after incubating with [$^{35}$S] Methionine containing medium. (The data shown is representative of at least 3 independent experiments.)**

### 3.5.2 Protein steady state level and degradation in mRNA are not different between SRC-1 P1272S and SRC-1 WT

The concentration of SRC-1 reflects the balance between its rate of synthesis and rate of degradation. For protein homeostasis the rate of protein synthesis may be adjusted to allow an adaptive change in the protein level. This might be detected by changes in steady state levels.

To test changes in steady state protein levels, either flag-tagged pSG5-SRC-1 WT or flag-tagged pSG5-SRC-1 P1272S was transfected into T47D, MCF-7, Ishikawa, or Hela cells and transfected SRC-1 levels were detected by immunoblotting for the flag-tag. SRC-1 P1272S protein level from two independent experiments was quantified and graphed as fold change over SRC-1 WT level. All tested cell lines showed no significant difference in SRC-1 protein levels between WT and P1272S (figure 23).

Due to the observed faster degradation in the presence of SRC-1 P1272S as a possible explanation for the described decrease in coactivity, SRC-1 P1272S protein levels were expected to be decreased compared to SRC-1 WT. Therefore, the result of no decrease in steady state levels with the SNP suggests that the protein synthesis of SRC-1 P1272S might be increased. However, this suggests that the ability of SRC-1 P1272S to reduce NR coactivation correlates with its degradation rate rather than its intracellular concentration.

To further determine if mRNA levels of SRC-1 P1272S compared to SRC-1 WT influence the observed protein characteristics, the mRNA degradation of SRC-1 WT and SRC-1 P1272S were compared. HEK293 cells were transiently transfected with a flag-tagged SRC-1 WT or flag- tagged SRC-1 P1272S construct. Actinomycin D was applied to the cells to inhibit transcription. RNA was isolated over a time course of four hours and subjected to qRT PCR using Flag- SRC-1 specific primer to detect only transfected SRC-1. No significant difference in mRNA stability between SRC-1 P1272S and SRC-1 WT could be detected (figure 24), suggesting that the differences in protein degradation between SRC-1 WT and SRC-1 P1272S might account for the differences in coactivation.

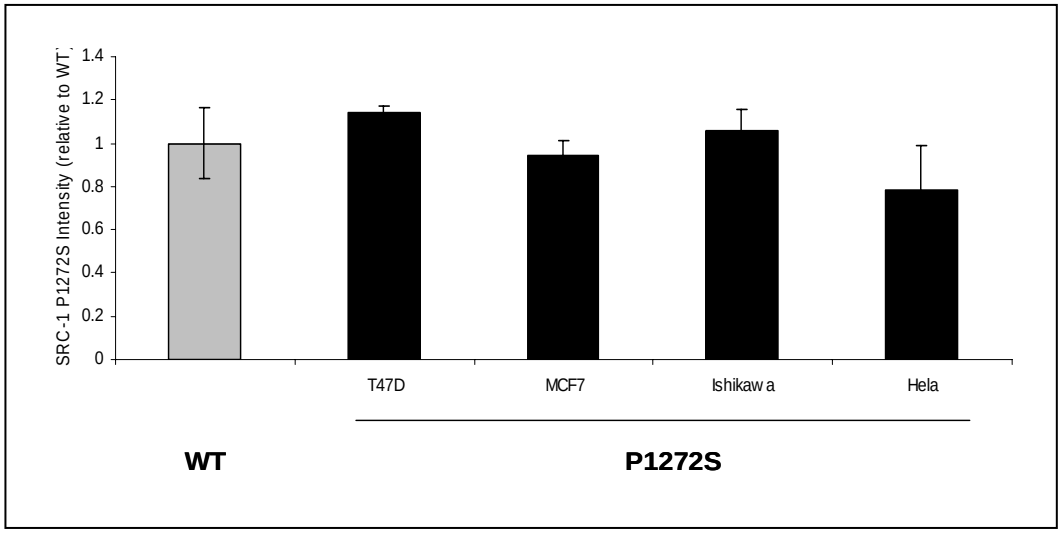



**Figure 23: SRC-1 steady state level.**

**Cells were transfected with 200ng of Flag-SRC-1 WT or Flag- SRC-1 P1272S. Protein was harvested and immunoblotted for SRC-1 using a Flag antibody. Protein expression was quantified. Quantitative expression of Flag-SRC-1 WT was set as one. Data is presented as fold change over Flag-SRC-1 WT. The data shown is representative of at least 3 independent experiments. Error bars represent standard deviation. Student's t-test was performed for Flag- SRC-1 WT versus Flag- SRC-1 P1272S.**

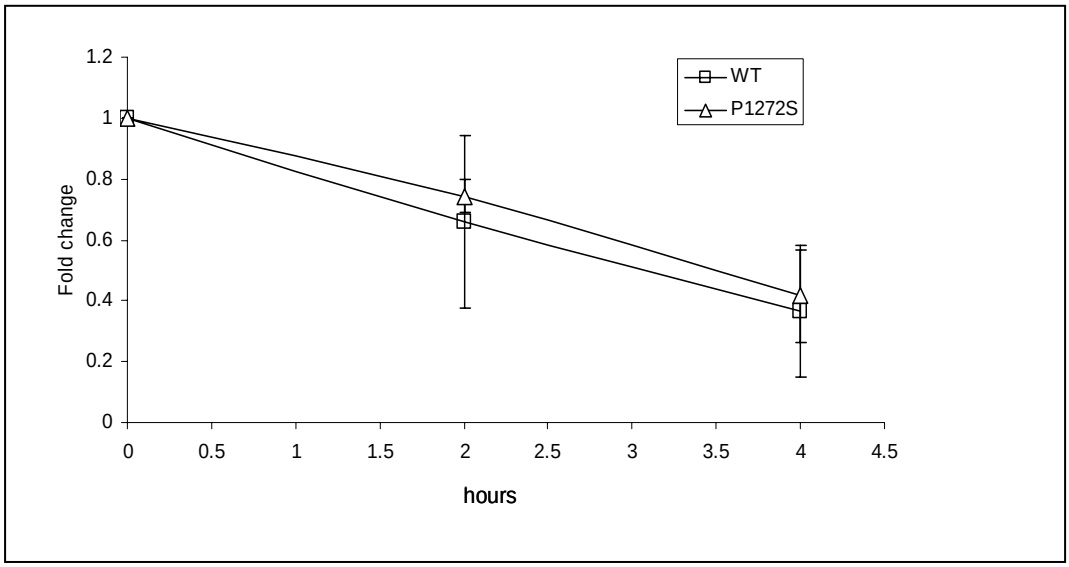



**Figure 24: mRNA degradation of SRC-1 P1272S compared to SRC-1 WT.**

**HEK293 cells were transfected with 200ng of Flag-SRC-1 WT or Flag- SRC-1 P1272S. Total RNA was harvested at 0, 2, and 4 hours to monitor SRC-1 mRNA degradation. RNA was subjected to qRT PCR using SRC-1 primers specific to Flag-SRC-1 and β-Actin specific primers to determine its expression. Data is expressed as SRC-1 mRNA abundance relative to β-Actin levels. The data is presented as fold over 0 timepoint for either SRC-1 WT or SRC-1 P1272S. Error bars represent standard deviation. Student's t-test was performed for Flag- SRC-1 WT versus Flag- SRC-1 P1272S. (The data shown is representative of 3 independent experiments.)**

### 3.5.3 Predicting phospho site changes in the presence of SRC-1 P1272S

Post-translational modifications, particularly in the form of phosphorylation, are critical for the activity of members of the p160 family of coactivators. Specifically, phosphorylation is involved in protein turnover and cellular localization of p160 family members[255]. Although most of this work has been done on SRC-3, the high sequence homology and partial functional redundancy within this family suggest that similar mechanisms are in place for all the family members[49].

Since the decrease in coactivation in the presence of SRC-1 P1272S might be due to faster degradation of the protein, we wanted to test if SRC-1 P1272S introduces changes in potential phosphorylation sites of SRC-1 that could explain faster degradation. Furthermore, the amino acid change from a proline to a serine with SRC-1 P1272S suggests that an introduction of a potential phosphorylation site might cause changes in protein and NR coactivity characteristics.

Using the program NetPhosK it was examined *in silico* if SRC-1 P1272S induced or destroyed any potential phosphorylation sites within SRC-1 (in collaboration with Ray-Chang Wu Ph.D, BCM, Houston, USA). NetPhosK is a program that predicts kinase specific potential eukaryotic protein phosphoylation sites (http://www.cbs.dtu.dk/services/NetPhosK). Indeed, we found that a cdc2 phosphorylation site is potentially created and a cdk5 phosphorylation site might be partially destroyed when SRC-1 P1272S is present (Table 3). Additionally, *in silico* analysis of the sequence suggests that the variant removes a potential Glycogen synthase kinase 3 (GSK3) target motif at amino acid 1275 (http://scansite.mit.edu/)[256]. Phosphorylation by GSK3 has been shown to be involved in modulating the activity and functional lifetime of another member of the SRC family, SRC-3[42].

| Kinase Site Probability | | | | |
|---|---|---|---|---|
| Site | Kinase | (WT) | (P1272S) | Difference |
| T1271 | **cdk5** | 60% | 23% | -37% |
| P1272 | **cdc2** | 0% | 52% | 52% |

**Table 4: Prediction of altered kinase binding sites in the presence of SRC-1 P1272S.**

**Scores represent predicted probability of a potential phosphorylation site for the specified kinase. The difference in these probabilities represents the probability of an altered phosphorylation (in collaboration with Ray-Chang Wu Ph.D, BCM, Houston, USA).**

### 3.5.4 SRC-1 alanine mutants for amino acid 1271, 1272, and 1275 show decreased ERα coactivity

In the presence of SRC-1 P1272S a potential cdc2 phosphorylation site is created at aa position 1272. A potential cdk5 phosphorylation site at aa position 1271 is partially destroyed when SRC-1 P1272S is present.

Additionally, the introduction of SRC-1 P1272S removes a potential GSK3 target motif. For GSK3 to phosphorylate a protein it requires a priming phosphorylation prior phosphorylation of an aa sequence of four aa C-terminal (+4) to the GSK3 target site (0)[42]. For SRC-1 S1275 is the potential consensus priming site, whereas T1271 is the potential target site that would be phosphorylated by GSK3. Both sides are predicted to be destroyed in the presence of SRC-1 P1272S.

To test whether potential changes in phosphorylation at these three sites will alter the ability of SRC-1 to coactivate ER, alanine mutants for aa 1271, 1272, and 1275 were generated using site directed mutagenesis.

To test whether the alanine mutants for those sites decrease ERα coactivity similarly to SRC-1 P1272S, transient estrogen-responsive luciferase reporter assays were conducted in Hela cells. Cells were transiently transfected with pSG5-SRC-1 WT, pSG5-SRC-1 P1272S, pSG5-SRC-1 T1271A, pSG5-SRC-1 P1272A, or pSG5-SRC-1 S1275A. Cells were cotransfected with a pCDNA3.1- ERα construct and treated with estrogen for 24 hours.

As shown before (figure 10) the transient transfection of WT SRC-1 resulted in an increase in ERα coactivation. Transfection of P1272S showed a significant diminished coactivation of ERα in the presence of the variant if compared to SRC-1 WT.

Transfection of the mutant SRC-1 T1271A, SRC-1 P1272A, or SRC-1 S1275A resulted in a significant decrease in coactivation compared to SRC-1 WT, which was similar to the lower coactivity observed with SRC-1 P1272S (figure 25).

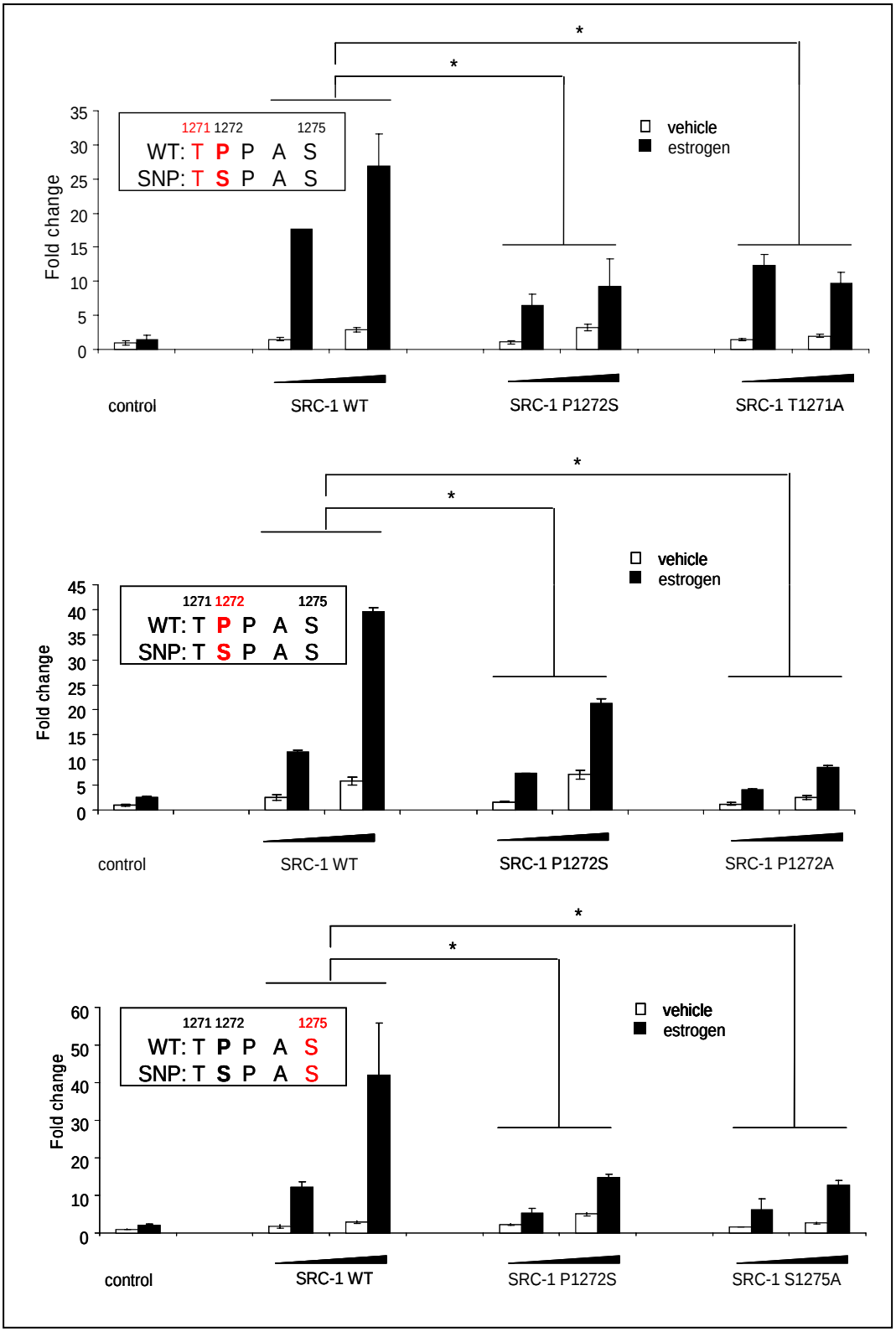

*
*
1271 1272 1275
WT: T P P A S
SNP: T S P A S
vehicle
estrogen
Fold change
35
30
25
20
15
10
5
0
control
SRC-1 WT
SRC-1 P1272S
SRC-1 T1271A
*
*
1271 1272 1275
WT: T P P A S
SNP: T S P A S
vehicle
estrogen
Fold change
45
40
35
30
25
20
15
10
5
0
control
SRC-1 WT
SRC-1 P1272S
SRC-1 P1272A
*
*
1271 1272 1275
WT: T P P A S
SNP: T S P A S
vehicle
estrogen
Fold change
60
50
40
30
20
10
0
control
SRC-1 WT
SRC-1 P1272S
SRC-1 S1275A

**Figure 25: SRC-1 alanine mutants show decreased ERα coactivity.**

**Hela cells were transiently transfected with 100ng ERE-TK-Luc reporter constructs and 200ng of expression constructs for pSG5-SRC-1 WT, pSG5-SRC-1 P1272S, pSG5-SRC-1 T1271A, pSG5-SRC-1 P1272A, or pSG5-SRC-1 S1275A. Cells were cotransfected with 5ng of a pCDNA3.1-ERα expression construct. Cells were treated with vehicle (ethanol) or estradiol ($10^{-8}$M) for 24h. Relative Luciferase Units were determined and normalized against total protein. The data is presented as fold over control, relative to untreated vehicle. The data shown are representative of at least 3 independent experiments for P1272S, T1271A, P1272A, and S1275A respectively. Error bars represent standard deviation. p –value was calculated using two-way ANOVA test (*, $p< 0.05$).**

### 3.5.5 SRC-1 alanine mutants for 1271, 1272, 1275 degrade faster than SRC-1 WT

As shown in figure 22 the decreased ERα coactivity in the presence of SRC-1 P1272S might be due in part to higher turn over rates and shorter half-life of the protein. Since the alanine mutants for aa 1271, 1272, and 1275 showed a similar decreased coactivation, we wanted to test how the mutants influence the half-life and turn over rates of the protein compared to SRC-1 WT and SRC-1 P1272S.

Hela cells were transiently transfected with constructs for SRC-1 WT, SRC-1 P1272S, SRC-1 T1271A, SRC-1 P1272A, or SRC-1 S1275S. Transfected cells were treated with either vehicle or estrogen over a time course of eight hours. Cycloheximide was added at the same time as vehicle and estrogen. Transfected SRC-1 protein was detected in westernblots using a SRC-1-antibody. Westernblots and subsequent quantification shows that SRC-1 P1272S degrades faster than SRC-1 WT in the absence and in the presence of estrogen (figure 26).

In the absence as well as in the presence of hormone all three alanine mutants degraded faster than SRC-1 WT (figure 26).

Together with the observation that SRC-1 P1272S degrades faster than SRC-1 WT under both conditions these results suggest that changes in potential phosphorylation sites might account for the differences in protein and coactivity characteristics. How a specific change in one potential phosphorylation site or the combination of several changes contributes to the decrease in NR coactivity in the presence of SRC-1 P1272S needs to be further elucidated.

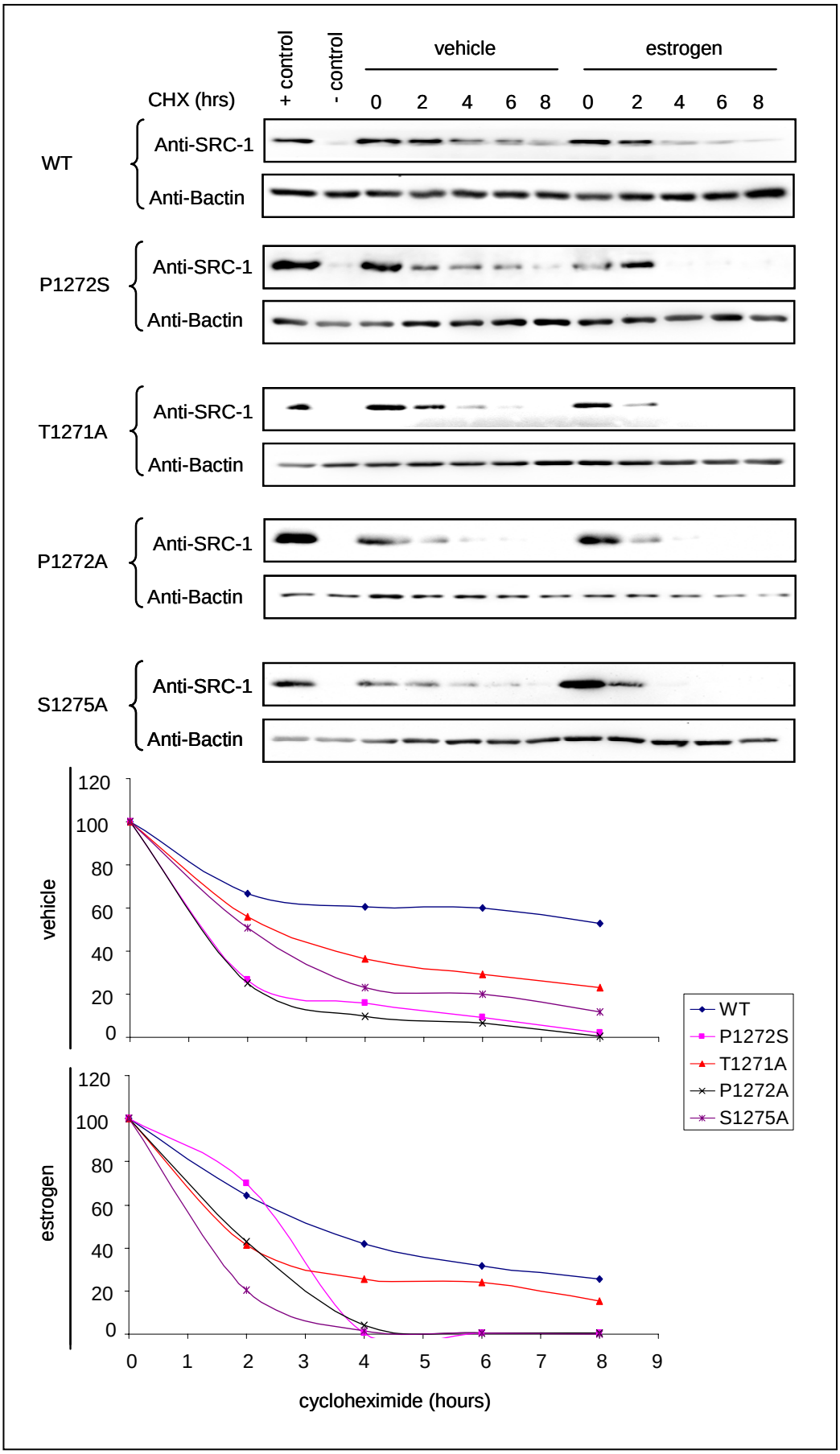

+ control
- control
vehicle
estrogen
CHX (hrs)
0 2 4 6 8 0 2 4 6 8
WT
Anti-SRC-1
Anti-Bactin
P1272S
Anti-SRC-1
Anti-Bactin
T1271A
Anti-SRC-1
Anti-Bactin
P1272A
Anti-SRC-1
Anti-Bactin
S1275A
Anti-SRC-1
Anti-Bactin
vehicle
120
100
80
60
40
20
0
estrogen
120
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9
cycloheximide (hours)
WT
P1272S
T1271A
P1272A
S1275A

**Figure 26: Degradation of SRC-1 alanine mutants.**

**Hela cells were transiently transfected with 200ng of expression constructs for SRC-1 WT, SRC-1 SNP, SRC-1 T1271A, SRC-1 P1272A, or SRC-1 S1275A. Cells were cotransfected with 5ng of an ERα construct. Cells were pretreated with cycloheximide (20µg/ml) for 30 min and subsequently treated with vehicle or estradiol (10nM) over the indicated time course. HEK293 protein lysate was used as positive control. A protein lysate obtained from untransfected Hela cells was used as a negative control. Protein was collected at indicated timepoints and immunoblotted for SRC-1 using a SRC-1 antibody. The graphic representation of the westernblotting results represents the intensity at the beginning of cycloheximide treatment (time 0) set as 1 for each treatment group (n=1).**

# 4 Discussion

In recent years it has become clear that genetic variation such as SNPs play a significant role in susceptibility to diseases or response to treatment. The completion of the International HapMap Project allows the identification of a continuously growing number of associations of genetic variations with common diseases and responses to drugs. SNPs in various genes have already been shown to contribute to a change in risk, outcome, or treatment efficiency for several diseases[184,206,207].

A functional SNP in exon 18 of SRC-1 was identified that occurs with a frequency between one and four percent in a normal population and is associated with a decrease in NR coactivity. This SNP is nonsynonymous and results in an amino acid change from proline to serine. Even though SRC-1 has been studied extensively and been proven to be an important NR coactivator for the pathology of osteoporosis[68,69,243] and breast cancer[65,66], the effect on disease susceptibility and treatment response of this SNP has not yet been studied.

SRC-1 P1272S is located in the AD2 of SRC-1, which contains an ERα binding motif and is known to be critical for its coactivator function[244]. *In silico* modeling studies predict that the P1272S substitution is probably damaging to the native structure of the protein (http://genetics.bwh.harvard.edu/pph/). *In vitro* studies of SRC-1 P1272S using ERE- luciferase assays expressing the variant resulted in a decrease in estrogen response when compared to WT. Furthermore, response to progesterone was attenuated in PRE-luciferase assays. This suggests for SRC-1 P1272S to play a role in hormone driven diseases. Our studies were focused primarily on the effects of SRC-1 P1272S on ER coactivity.

To test the role of SRC-1 P1272S for breast cancer susceptibility, human cancer cell lines and clinical samples for SRC-1 P1272S were genotyped.

In the presence of SRC-1 P1272S estrogen-dependent ERα coactivation is diminished, suggesting that carrying the SNP might decrease the risk for developing hormone-dependent breast cancer and could serve as a prognostic marker. Therefore, ER positive breast cancer cells would not be expected to carry SRC-1 P1272S. SRC-1 P1272S does

not occur in any of the screened breast cancer cell lines. Since only cancer cell lines were genotyped for SRC-1 P1272S, the hypothesis that the SNP might exert a protective effect on developing hormone-dependent breast cancer needs to be studied by comparing these results with genotyping for SRC-1 P1272S in normal cell lines.

Out of 28 cancer cell lines, only the colon cancer cell line Caco-2 was identified to be heterozygous for the SNP. Reduced activity of PPARγ, which is coactivated by SRC-1 is associated with a higher risk of developing colon cancer[257]. How SRC-1 P1272S alters PPARγ coactivity was not tested but it is possible that in the presence of the SNP PPARγ activity is diminished. Thus, carrying SRC-1 P1272S might increase the risk for colon cancer. Since the other genotyped colon cancer cell line (LS-174T) did not show this variation in SRC-1, this hypothesis needs further evaluation.

To further test if carrying SRC-1 P1272S results in a decreased risk for developing breast cancer, a case- control study for association with breast cancer risk was performed. SRC-1 P1272S did not show an association with breast cancer risk in the studied population. SRC-1 P1272S occurs in a normal population with a frequency of only one to four percent. The low frequency of this SNP might explain why no association was detected.

The SERM tamoxifen is a standard treatment for ERα positive breast tumors due to its antagonistic effect in the breast. SRC-1 protein was significantly associated with insensitivity to endocrine treatment[66]. SERMs have tissue-specific mixed agonist/antagonist activity, and SRC-1 overexpression can result in increased agonist, and decreased antagonist activity in certain tissues such as bone[35] and breast[40]. Further, clinical studies to determine the effect of SRC-1 P1272S on breast cancer recurrence or survival in response to tamoxifen should be conducted.

Osteoporosis is one of the major implications associated with estrogen deficiency in postmenopausal women. Considering the prolonged population lifetime, the number of women with osteoporosis will continue to increase, and it has been estimated that costs related to hip fracture will double during the next 25 years. Thus, there is a critical need for further genetic investigations to identify new possibilities for discovering risk factors and early prevention of this condition. Postmenopausal women can decrease their risk for developing osteoporosis by taking SERMs like tamoxifen[258]. There is evidence that SRC-1 may play a role in the response to tamoxifen[40]. Deletion of SRC-1 in mice results in decreased hormone response in bones[69,243].

Breast cancer patients carrying SRC-1 P1272S had a significant decrease in bone mineral density after 12 months of tamoxifen treatment, presumably reflecting tamoxifens loss of

agonist activity on bone formation. Subgroup analysis concentrated on BMD of combined pre- and postmenopausal women which were only treated with tamoxifen and received no additional chemotherapy. For women carrying SRC-1 WT tamoxifen treatment for 12 months is expected to increase BMD in postmenopausal women, whereas premenopausal women show decreased BMD at the same time[259]. The distribution between pre- and post menopausal women was equal within groups for SRC-1 WT or SRC-1 P1272S. Considering our study population this may explain, the observed small decrease of BMD in women carrying SRC-1 WT. This indicates that the loss in BMD in premenopausal women is even stronger and postmenopausal women do not benefit from tamoxifen treatment if carrying SRC-1 P1272S in respect to BMD. *In vitro* it was shown that SRC-1 P1272S decreases tamoxifen's agonistic activity. Our data suggest that women harboring this variation in the SRC-1 locus may require close monitoring and early intervention with alternative bone-preserving agents. Final confirmation of the critical importance of this SNP is awaiting genotyping in additional cohorts, but the fact that similar findings were made in tissue culture, animal models, and clinical samples strongly argue for a critical role of SRC-1 P1272S in the response of skeletal cells to ligands.

Estrogen can directly act on osteoclasts and alter bone resorption by inducing osteoclast apoptosis[151]. To examine the effect of a decrease in ER coactivation by SRC-1 P1272S on bone maintenance, first an apoptosis assay in skeletal cell cultures derived from SRC-1 ko mice was performed. Cells containing pre-osteoclasts and mature osteoclasts were obtained by differentiating bone marrow cells with M-CSF and RANKL. Since estrogen strongly induces osteoclast apoptosis, it suggests that a major mechanism for bone maintenance in premenopausal women is the suppression of osteoclast numbers[137]. Estrogen failed to induce apoptosis in skeletal cell cultures in the presence of SRC-1 P1272S, whereas SRC-1 WT seems to be necessary to induce apoptosis in the presence as well as in the absence of estrogen. Interestingly, osteopenia with high bone turnover is observed in SRC-1 ko mice, an effect that is similar to that seen in ovariectomized WT mice[69,243]. Exogenous estrogen is able to reverse the bone loss in ovariectomized WT mice, but not in ovariectomized SRC-1 ko mice, supporting the critical role of SRC-1 in estrogen-dependent bone maintenance[69,243].

To determine how a decrease in ER coactivity caused by SRC-1 P1272S leads to an increase in osteoclast apoptosis, that might explain the observed bone loss *in vivo,* the two target genes TIEG and FasL were studied, which were shown to be involved in estrogen dependent bone maintenance[168,169].

Apoptosis of osteoclasts can be induced by a paracrine mechanism in which estrogen affects osteoclast survival through the upregulation of FasL in osteoblasts[168]. A consistent SRC-1 dependent estrogen-induction of FasL compared to control could not be observed. Therefore, the role of FasL regulation by SRC-1 and the decreased apopoptosis rate of osteoclasts with SRC-1 P1272S in the presence of estrogen need to be further elucidated.

As reported previously[169] SRC-1 is essential for estrogen induction of TIEG in ERβ containing osteoblast derived cells. This implies a specific role of SRC-1 on bone maintenance through regulation of TIEG.

Overexpression of TIEG enhances TGF-β functions, which is an important factor for bone remodeling[251]. TGF-β produced by osteoblasts can stimulate osteoclast apoptosis. SRC-1 P1272S shows decreased TIEG expression compared to SRC-1 WT in the presence of estrogen as well as tamoxifen. Therefore, our observation of decreased osteoclast apoptosis in the presence of SRC-1 P1272S might be due to decreased TIEG expression and subsequently decreased TGF-β function.

These results suggest that carrying the SRC-1 P1272S variant results in a decreased hormone response, decreased TIEG levels and potentially in decreased TGF-β signaling. This could lead to decreased osteoclast apoptosis and cause a decrease in BMD (figure 14).

Additionally, to TGF-β's function of inducing osteoclast apoptosis it plays an essential role in osteoblast differentiation as well as development and remodeling of bone[253,260]. As demonstrated earlier SRC-1 WT increases TIEG levels in response to estrogen and tamoxifen. Osteoblasts derived from SRC-1 ko mice confirmed that SRC-1 is needed to induce TIEG in response to estrogen and tamoxifen.

Therefore, the role of SRC-1 on osteoblast proliferation and differentiation was tested. To determine the effect of SRC-1 on osteoblast proliferation a MTS assay was performed. It revealed slower proliferation of osteoblasts derived from SRC-1 ko mice if compared to osteoblasts derived from SRC-1 WT or SRC-1 heterozygous littermates. Further, estrogen and tamoxifen did not increase proliferation rates in SRC-1 ko osteoblast in contrast to SRC-1 WT and SRC-1 het osteoblasts. This indicates that SRC-1 seems to play an important role in osteoblast proliferation. To test how SRC-1 affects osteoblast differentiation it was tested if lacking SRC-1 alters the expression of the important osteoblast differentiation marker ALP in osteoblasts derived from mice. This revealed that SRC-1 seems to be needed for hormone-dependent differentiation of osteoblasts characterized by induction of ALP. Interestingly, TIEG ko mice also show decreased

expression of ALP[174]. These results suggest that SRC-1 is needed for proper osteoblast proliferation and differentiation potentially through its effect on TIEG.

The activity of SRC-1 is in part regulated via its degradation through the proteasome pathway[254]. It was shown that SRC-1 P1272S degrades faster than SRC-1 WT. Faster turn over rates of SRC-1 P1272S might suggest an increase in protein synthesis to maintain the protein level. Higher rates of protein synthesis were not detectable by increased mRNA levels or protein steady state levels. Therefore, the decrease in coactivation in the presence of SRC-1 P1272S might be due to faster degradation of the protein.

The process of nuclear receptor-induced coactivator recruitment and initiation of transcription happens in a cyclic fashion. This cycling is also regulated by phosphorylation of SRCs[58,261]. Posttranslational modifications, particularly phosphorylation is critical for the activity of members of the p160 family of coactivators. Phosphorylation is involved in protein turnover and cellular localization of p160 family members[42]. Although most of this work has been done on SRC-3, the high sequence homology and partial functional redundancy within this family suggest that similar mechanisms are in place for SRC-1[49].

If SRC-1 P1272S introduces changes in potential phosphorylation sites of SRC-1 that might explain the faster degradation was tested. The major phosphorylation sites identified in the AD2 of SRC-1 are threonine 1179 and serine 1185[58]. Threonine 1179 contains a perfect consensus sequence for the MAPK family extracellular signal regulated kinases (Erk-1 and Erk-2). Erk-2 phosphorylates threonine 1179 and serine 1185 *in vitro*, suggesting the importance of this pathway for SRC-1 regulation. No phosphorylation sites surrounding SRC-1 P1272S were identified what might be due to the high content of prolines in close proximity to amino acid 1272[58].

*In silico* analysis revealed that in the presence of SRC-1 P1272S a potential cdc2 site is created at aa 1272. If introducing a potential phosphorylation site at aa 1272 causes decreased SRC-1 coactivity, it would be expected that mutating aa 1272 to alanine would result in increased activation, which would be comparable to SRC-1 WT activity. ERE-Luciferase assays showed that transfecting P1272A mutant resulted in a decreased coactivity comparable to SRC-1 P1272S. Further, experiments to compare the degradation of P1272A with SRC-1 WT and SRC-1 P1272S showed that P1272A degrades faster than SRC-1 WT and comparable to SRC-1 P1272S in the absence and in the presence of estrogen.

Proline residues are widely recognized as p1aying a special role in the folding and unfolding transitions of globular protein molecules[262]. Breaking hydrogen bonds around proline plays a role in destabilizing alpha-helical conformations[263]. Proline blocks rapid folding [262] that might be necessary for proper function in a proline rich region such as the SRC-1 AD2. Introducing a change from a proline to a serine such as in SRC-1 P1272S could indicate a potential change in protein folding causing decreased activation by disrupting proper interaction with nuclear receptors or other proteins to activate transcription.

Alanine mutants are used as a tool to determine the function of proline for protein folding[264]. SRC-1 P1272A provides a tool to test if changes in folding can account for the decreased coactivity and the faster protein degradation. The AD2 of SRC-1 including the SNP at aa 1272 is considered to be proline-rich and therefore exists in an unfolded state (personal communication with Ma Peng Ph.D, BCM, Houston, USA). Such native unfolded sequences achieve folded structures to carry out their functions[265]. Induced folding results in enhanced interactions of glucocorticoid receptor with coactivator proteins[266]. Since serine is an amino acid that can occur in an α-helix as secondary structure, a SNP that changes proline to serine might introduce significant changes in protein folding. This can result in enhancing its ability to interact with other proteins such as the methyltransferase CARM1. Interestingly, methylation of SRC-3 by CARM1 correlates with decreased ERα-mediated transcription[87] and promotes degradation of the protein[88]. How changes in protein folding caused by SRC-1 P1272S specifically account for decreased ERα coactivation, faster degradation, and interaction and subsequent methylation by CARM1 is subject to further investigation.

*In silico* analysis also revealed that in the presence of SRC-1 P1272S a potential cdk5 site at aa 1271 is partially destroyed. Additional analysis of the sequence suggests that the variant removes a potential GSK3 target motif at aa 1275 (http://scansite.mit.edu/). Intriguingly, phosphorylation by GSK3 has been shown to be involved in modulating the activity and functional lifetime of SRC-3[42]. For GSK3 to phosphorylate a protein it requires a priming phosphorylation of an amino acid four positions C-terminal (+4) to the GSK3 target site (0)[42]. For SRC-1 S1275 is a potential consensus GSK3 priming site, whereas T1271 is the potential target site that would be phosphorylated by GSK3. Both sites are potentially destroyed in the presence of SRC-1 P1272S.

How changes in potential phosphorylation sites at aa 1271 and 1275 alter ERα coactivity using alanine mutants at these sites was tested. Transfection of the individual alanine

mutants resulted in a significant decreased ERα coactivity. Additionally, T1271A and S1275A degraded faster than SRC-1 WT mimicking turn-over characteristics of SRC-1 P1272S. This suggests that losing a potential GSK3 priming site at aa 1275 and subsequently losing a potential GSK3 phosphorylation site at aa 1271 in the presence of SRC-1 P1272S could cause the observed decrease in coactivity and faster protein degradation. How differences in potential phosphorylation of SRC-1 by GSK3 in the presence of SRC-1 P1272S are causing changes in coactivator activity and degradation needs further evaluation.

Furthermore, aa 1271 resembles a potential consensus phosphorylation site for MAPK (TPXXS). Phosphorylation by the MAPK pathway plays an important role in the phosphorylation of SRC-1[58]. Mutation of MAPK target sites aa 1179 and aa S1185 to alanine resulted in up to a 50% decrease in coactivation during both ligand-independent activation and ligand-dependent activation[58]. This might suggest that losing the potential consensus phosphorylation site for MAPK in the presence of SRC-1 P1272S results in the observed decrease in ERα coactivation. How potential phosphorylation by MAPK alters coactivity and degradation of SRC-1 WT or SRC-1 P1272S needs to be elucidated.

Importantly, decreasing SRC-1 expression in the uterine cell line Ishikawa converted tamoxifen to an antagonist[40]. At the same time, overexpression of SRC-1 in MCF-7 cells converted tamoxifen to an agonist[40]. Tamoxifen resistant tumors show that the drug acquires agonistic properties towards ERα[267]. Altered activity of coactivator proteins at the ER–ERE complex may be important in the alteration of the agonist/antagonist profile of SERMs in resistant tumors[66]. The specificity and activity of SRC-1 is thought to be regulated by intracellular signaling cascades, including phosphorylation via PKA[58,267] or the MAPK pathway[58].

PKA mediates tamoxifen resistance by phosphorylating ERα, which causes conformational changes in ERα after binding tamoxifen and therefore switches tamoxifen from an antagonist to an agonist[268]. Specifically, PKA mediated phosphorylation alters the orientation between ERα and SRC-1 in tamoxifen-treated cells without changing the overall binding between ERα and SRC-1 leading to enhanced estrogen-dependent transcriptional activity in the presence of tamoxifen[267].

Growth factor induced MAPK activity cannot only phosphorylate ER but also its coactivator proteins SRC-1 and SRC-3[59]. Significant correlations between coactivator proteins and resistance to endocrine treatment in patients who overexpress the tyrosine kinase receptor HER2 have been reported[66]. SRC-1 was associated with HER2

expression[66] supporting previous molecular *in vitro* data linking SRC-1 and HER2 to human breast cancer[269].

Further, Osborne *et al.* found that tumours with high expression rates of both SRC-3 and HER2 have a poor response to tamoxifen[136]. In human breast cancer, HER2 is associated with disease progression and resistance to endocrine treatment. The overexpression of HER2 and SRC-3[35] or SRC-1[40] may significantly enhance the agonist activity of tamoxifen and, therefore, reduce the antitumor activity of tamoxifen in patients with breast cancer.

Altogether, phosphorylation of ERα by PKA or phosphorylation of SRC-1 by MAPK might lead to activation of SRC-1 in the presence of tamoxifen and subsequently enhance the ability of SRC-1 to induce tamoxifen's agonistic activity. These findings suggested that tamoxifen resistance in breast tumors could be due to the overexpression of SRC-1 leading to conversion of tamoxifen from an antagonist to an agonist. Losing a MAPK phosphorylation site reduces SRC-1 activity[58]. Therefore, the prediction of losing a potential MAPK phosphorylation site in the presence of SRC-1 P1272S might result in the observed decrease of coactivator activity and subsequently could account for a decrease in risk for tamoxifen resistance. The effect of SRC-1 P1272S on tamoxifen resistance needs to be elucidated. Altogether, it could be shown that the identification of genetic variations such as SRC-1 P1272S has the potential to function as a marker for individual treatment responses and could be a key to personalized medicine.

# 5 Materials and Methods

## 5.1 Materials

Unless otherwise specified all primers used were obtained by Sigma-Genosys (Sigma-Aldrich, St. Louis, MO).

| | **Catalogue #** | **Company** |
|---|---|---|
| [35S]- Methionine, 500µCi (18.5MBq) | NEG009A500UC | PerkinElmer Life And Analytical Sciences, Inc., Waltham, MA |
| 4-OH Tamoxifen | H7904 | Sigma-Aldrich, St. Louis, MO |
| Actinomycin D | A1410 | Sigma-Aldrich, St. Louis, MO |
| α-MEM | 32561037 | Invitrogen, Carlsbad, CA |
| Anti-β-actin antibody | A3853 | Sigma-Aldrich, St. Louis, MO |
| Anti-Flag antibody | F1804 | Sigma-Aldrich, St. Louis, MO |
| Anti-SRC-1 (128E7) antibody | 2191 | Cell Signaling Technology, Inc., Danvers, MA |
| BCA protein assay | 23221 | Thermo Scientific, Rockford, IL |
| CellTiter 96® AQueous One Solution Cell Proliferation Assay | G3580 | Promega Corporation, Madison, WI |
| Charcoal treated fetal bovine serum (CSS) | SH30068.03 | Thermo Scientific, Rockford, IL |
| Collagenase P | 11213857001 | Roche Applied Science, Indianapolis, IN |

| | | |
|---|---|---|
| Cycloheximide | C7698 | Sigma-Aldrich, St. Louis, MO |
| Dulbecco's Modified Eagle Medium (DMEM) | 12430-104 | Invitrogen, Carlsbad, CA |
| DMEM without L-Methionine or L-Cysteine | 21013-024 | Invitrogen, Carlsbad, CA |
| DNeasy® Blood and Tissue Mini Kit | 69504 | Qiagen, Valencia, CA |
| Dual Luciferase reporter assay system | E1910 | Promega Corporation, Madison, WI |
| ECL mouse IgG HRP-linked AB | NA931 | Amersham, Piscataway, NJ |
| ECL rabbit IgG HRP-linked AB | NA934 | Amersham, Piscataway, NJ |
| Estradiol | E8875 | Sigma-Aldrich, St. Louis, MO |
| Fetal Bovine Serum (FBS) | SH30070.03 | Thermo Scientific, Rockford, IL |
| Immun-Blot PVDF Membrane | 162-0177 | Biorad, Hercules, CA |
| Improved Modified Eagle Medium (IMEM) | A10488-01 | Invitrogen, Carlsbad, CA |
| Lipofectamine 2000 | 11668-019 | Invitrogen, Carlsbad, CA |
| M-CSF | 416-ML-010 | R&D Systems, Inc., Minneapolis, MN |
| Opti-MEM® | 11058-021 | Invitrogen, Carlsbad, CA |
| PBS | 10010023 | Invitrogen, Carlsbad, CA |
| Penicillin- Streptomycin – Glutamine | 10378-016 | Invitrogen, Carlsbad, CA |
| p- Formaldehyde | F8775 | Sigma-Aldrich, St. Louis, MO |
| Progesterone | P0130 | Sigma-Aldrich, St. Louis, MO |

| | | |
|---|---|---|
| Protein G Sepharose®4B | 10-1241 | Invitrogen, Carlsbad, CA |
| QuickChange® Sitedirected Mutagenesis Kit | 200521 | Stratagene, La Jolla, CA |
| RANKL | 315-11 | Peprotech, Rocky Hill, NJ |
| Sodiumdodecylsulfate | L4390 | Sigma-Aldrich, St. Louis, MO |
| TRAP Staining Kit | 387A | Sigma-Aldrich, St. Louis, MO |
| Trypsin | 25200-056 | Invitrogen, Carlsbad, CA |
| TUNEL assays | 11684795910 | Roche Applied Science, Indianapolis, IN |
| Tween20 | BP337-500 | Fisher Scientific, Pittsburgh, PA |
| VECTASHIELD® with DAPI | H-1200 | VECTOR LABORATORIES, INC., Burlingame, CA |

## 5.2 Methods

**Cell culture.** Unless otherwise specified cell lines were obtained by ATCC (American Type Culture Collection, Manassas, VA, USA). HEK293, MCF-7, Hela, Ishikawa, T47D, and HepG2 cells were maintained in DMEM (Invitrogen, Carlsbad, CA) culture media supplemented with 5% FBS (Thermo Scientific, Rockford, IL), and 1% Penicillin-Streptomycin-Glutamine (Invitrogen, Carlsbad, CA). U2OS-Flag-ERα and U2OS-Flag-ERβ cells were provided by Dr. D. Leitman (UCSF, San Francisco, CA) and cultured in IMEM (Invitrogen, Carlsbad, CA) culture media supplemented with 10% FBS (Thermo Scientific, Rockford, IL), and 1% Penicillin-Streptomycin-Glutamine. Prior to hormone treatment all cells were maintained in IMEM (Invitrogen, Carlsbad, CA) supplemented with 5% charcoal-dextran stripped serum (CSS) (Thermo Scientific, Rockford, IL), and 1% Penicillin-Streptomycin-Glutamine (Invitrogen, Carlsbad, CA).Cells were incubated at 37°C and 5% $CO_2$.

**SRC-1 sequencing**. Target sequence obtained from NCBI consisting of all exons, 500bp of proximal promoter, and 25bp of flanking introns from SRC-1 was submitted for primer design and Sanger sequencing to Polymorphic DNA Technologies Inc. (Alameda, CA).

DNA from 96 samples (48 Caucasian American, 48 African American) obtained from the Coriell Institute (Camden, NJ, USA) (sample sets: HD100CAU and HD100AA) was sequenced in both directions and aligned to the NCBI reference sequence and previously reported SNPs in the dbSNP data base. These samples were collected and anonymized by the National Institute of General Medical Sciences. Visual inspection of chromatograms was conducted for heterozygous base calls. Alignment was performed by Jay Wang M.D. (BCM, Houston, USA) in collaboration with COBRA.

**Plasmids and mutagenesis.** The pSG5 – SRC-1 WT expression plasmid was provided by Dr. C. Smith (Baylor College of Medicine, Houston, TX). The SRC-1 variants P1272S (Jay Wang M.D.,BCM, Houston, USA), T1271A, P1272A, and S1275A were generated by mutagenesis using the QuickChange® Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA) following the manufacturer's protocol. Primer sequences are listed in table 5.The pcDNA3.1-HA-ERα construct was provided by Dr. A. Lee (BCM, Houston, USA). The pCR3.1-PR and the PRE-tata-LUC constructs were provided by Dr. N. Weigel (BCM, Houston, USA).

| | **Forward primer sequence** | **Reverse primer sequence** |
|---|---|---|
| P1272S | 5'-ttctcttctccagcaagcttc acctgcctccgggtatcag-3' | 5'-gtgatacccggaggcaggtga agcttgctggagaagaaag-3' |
| T1271A | 5'-gagttctcttctccagcaagct ccacctgcctccgggtatcag-3' | 5'-ctgatacccggaggcaggtgga gcttgctggagaagagaactc-3' |
| P1272A | 5'-ttctcttctccagcaagctgc acctgcctccgggtatcag-3' | 5'-ctgatacccggaggcaggtg cagcttgctggagaagagaa-3' |
| S1275A | 5'-gcaaactccacctgccgccgggt atcagtcaccagacatgaaggc-3' | 5'-gccttcatgtctggtgactgata cccggcggcaggtggagtttgc-3' |

**Table 5: Primer sequences for mutagenesis.**

**Transient transfection.** Transfection of plasmids was performed using Lipofectamine 2000 reagent from Invitrogen (Carlsbad, CA) according to the manufacturer's protocol.

**Luciferase reporter assay.** Cells were plated in six-well plates at $1.5x10^5$ cells per well in DMEM (Invitrogen, Carlsbad, CA) + 5% FBS (Thermo Scientific, Rockford, IL) 48

hours prior to transfection. The medium was changed to IMEM (Invitrogen, Carlsbad, CA) supplemented with 5% CSS (Thermo Scientific, Rockford, IL) 24h prior to transfection, and transfections were carried out using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) in OPTIMEM (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. For treatment with estradiol (Sigma, St. Louis, MO) or tamoxifen (Sigma, St. Louis, MO) in the reporter assay, estradiol ($10^{-8}$M) or tamoxifen ($10^{-6}$M) was added to the cells 24 hours posttransfection and incubated for another 24 hours (37°C; 5% $CO_2$). Cells were harvested and luciferase activity was determined and normalized against total protein.

**Human SRC-1 genotyping.** For the genotyping studies, germline DNA was extracted from the leukocyte portion of whole blood using a DNeasy® Blood and Tissue Mini Kit (Qiagen, Valencia, CA). For genotyping cell lines genomic DNA was extracted using a DNeasy® Blood and Tissue Mini Kit (Qiagen, Valencia, CA). SRC-1 P1272S (rs1804645) variant alleles were genotyped with a Taqman Allelic Discrimination Assay (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The digested polymerase chain reaction products were then analyzed with an Agilent 2100 Bioanalyzer (Agilent Technologies, Rockville, MD). Genotyping was performed by COBRA.

## Study population.

*Case-control study for breast cancer risk in a German-Polish population.*

The cases were unrelated, female, BRCA1/2 mutation–negative individuals with breast cancer. Breast cancer cases were selected according to the criteria used for BRCA1 and BRCA2 mutation screening. Using these criteria, familial and early-onset cases were accumulated, which are more likely to be due to a genetic cause. The controls were chosen from the same geographic area and ethnic background as the breast cancer cases. The analysis was done using genomic DNA from 1218 breast cancer cases and 1509 controls. The study was approved by the ethics committee of the University of Heidelberg (Heidelberg, Germany). The study was conducted by the laboratory of Dr. Barbara Burwinkel at the DKFZ, Heidelberg, Germany.

*COBRA tamoxifen response association study.*

The registry protocol was approved by the institutional review boards of all participating sites and registered on www.clinicaltrial.gov (NCT00228930). All patients provided informed written consent before entry. Eligible women were recruited into a prospective

cohort registry from three breast cancer clinics—the Lombardi Comprehensive Cancer Center at Georgetown University Medical Center (Washington, DC); the Breast Oncology Program at the University of Michigan Comprehensive Cancer Center (Ann Arbor, MI); and the Indiana University Cancer Center (Indianapolis, IN). Premenopausal and postmenopausal women (aged ≥18 years) at high risk for breast cancer, or with newly diagnosed breast cancer who were starting tamoxifen as standard adjuvant therapy were included in this registry. Patients were enrolled after they had completed all primary surgery, radiation, and adjuvant chemotherapy. Since chemotherapy was a strong confounding factor for BMD in this trial (Henry L. *et al*. unpublished data), the analysis was limited to patients treated exclusively with tamoxifen. Hip and lumbar BMD was measured by DXA scanning before the start of tamoxifen treatment ("baseline") and after 12 months of treatment (PS207714 and PS207749; www.pharmgkb.org). The study and analysis was conducted by COBRA.

## Statistical analysis.

*Case-control study for breast cancer risk in a german-polish population.*

Odds ratios (OR) and 95% confidence intervals (95% CI) were calculated for genotype frequencies between breast cancer cases and controls using logistic regression adjusting for country. Power calculations were determined using power and sample size calculator software PS version 2.1.31 (http://www.mc.vanderbilt.edu/prevmed/ps/). Calculations for Hardy-Weinberg equilibrium were carried out using the Hardy-Weinberg equilibrium tool offered by the Institute of Human Genetics, Technische Universität, Munich, Germany (http://ihg.gsf.de/cgibin/hw/hwal.pl). The statistical analysis was performed by Dr. Barbara Burwinkel's group.

*COBRA tamoxifen response association study.*

Associations between SRC-1 genotypes P1272S and baseline lumbar and hip BMD were examined in each menopausal group. The comparisons were performed using linear regression within each menopausal status. Associations between SRC-1 genotypes and the percent changes in lumbar and hip BMD from baseline to month 12 were assessed by using a general linear model (GLM) and adjusted for center. GLM was performed using the SAS procedure (PROC GLM, SAS v9.1.3). For post-hoc comparisons, the adjusted means between all pairs of three genotypes were compared while controlling for overall alpha. For all analyses, a p-value of equal to or less than 0.05 was considered statistically significant. The statistical analysis was performed by Dr. Lang Li (Indiana University School of Medicine, Indiana, IN).

**Isolating osteoclasts from SRC-1 ko mice.** Bone marrow cells were isolated from femur and tibia of SRC-1 ko mice. Cells were plated in six-well tissue culture plates containing IMEM Invitrogen, Carlsbad, CA) with 10% FBS (Thermo Scientific, Rockford, IL) and 10ng/ml M-CSF (R&D Systems, Minneapolis, MN). After incubation for 48h (37°C; 5% $CO_2$), cells were cultured for 13 days in the presence of 10ng/ml M-CSF and 100ng/ml RANKL (Peprotech, Rocky Hill, NJ) to generate osteoclasts.

**Trap staining.** To test for osteoclastic activity TRAP staining was performed according to the manufacturer's protocol (Sigma-Aldrich, St. Louis, MO).

**TUNEL assays.** The differentiated SRC-1 ko skeletal cell cultures were transfected with pSG5 (empty vector), pSG5-SRC-1 WT or pSG5-SRC-1 P1272S expression constructs and treated with estrogen ($10^{-8}$M) for 16 hours. Cells were fixed with 4% paraformaldehyde and TUNEL assay (Roche Applied Science, Indianapolis, IN) was performed according to the manufacturer's protocol. Apoptosis was detected by determining the ratio of TUNEL-positive cells and DAPI-counterstained cells via fluorescence microscopy. The experiment was performed in triplicates.

**Quantitative PCR analysis.** Total RNA was isolated using the QIAShredder and Rneasy kit (Qiagen, Valencia, CA). The mRNA was reverse transcribed into cDNA by SuperScript III First-Strand Synthesis system for RT-PCR (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol.

Primer sequences are listed in Table 6.

| **Primer** (obtained from Sigma Genosys, St. Louis, MO) | **Forward primer sequence** | **Reverse primer sequence** |
|---|---|---|
| Alkaline phosphatase (mouse) | 5'-aatgaggtcacatccatcctgc-3' | 5'-tagctgatatgcgatgtccttgc-3' |
| β-actin (human) | 5'-ccctggcacccagcac-3' | 5'-gccgatccacacggagtac-3' |
| β-actin (mouse) | 5'-tcgtgcgtgacatcaaagaga-3' | 5'-ccgctcgttgccaatagtg-3' |
| ERα (mouse) | 5'-ctagcagatagggagctggttca-3' | 5'-ggagattcaagtccccaaagc-3' |
| ERβ (mouse) | 5'-atgactatatctgtccagccacg-3' | 5'-ctcagagagttcagcagtagc-3' |
| FasL (human) | 5'-ggcccatttaacaggcaagtc-3' | 5'-ggccacccttcttatacttcac-3' |
| TIEG (human) | 5'-gccaaccatgctcaacttcg-3' | 5'-tgcagttttgttccaggaatacat-3' |
| TIEG (mouse) | 5'-gtctcagtgctcccgtctgt-3' | 5'-ccaccgcttcaaagtcactc-3' |
| SRC-1 (human) | 5'-tgaaagtggaaaagaaagaacagatg-3' | 5'-gtcaagtcagctgtaaactggc-3' |
| SRC-1 (mouse) | 5'-tatctctccagcccatggtgt-3' | 5'-caaagttcccttggttgttgc-3' |

**Table 6: Primer sequences for qRT-PCR.**

**Isolation of osteoblasts.**

Calvaria were obtained from offspring of heterozygous matings 5 days after birth and washed three times in cold PBS (Invitrogen, Carlsbad, CA). Each calvaria was placed in a well of a six-well plate and incubated for 20 min in αMEM (Invitrogen, Carlsbad, CA) supplemented with 0.1 mg/ml collagenase P (Roche Applied Science, Indianapolis, IN)

and 0.04% trypsin (Invitrogen, Carlsbad, CA) at 37ºC and shaked every five minutes for 20 seconds. The released cells were discarded. This step was repeated once and the calvaria were transferred to αMEM (Invitrogen, Carlsbad, CA) supplemented with 0.2 mg/ml collagenase P (Roche Applied Science, Indianapolis, IN) and 0.04% trypsin (Invitrogen, Carlsbad, CA). Calvaria were dissected and digested for 1 h at 37ºC with shaking every five minutes. Digestion was stopped by the addition of αMEM (Invitrogen, Carlsbad, CA)/ 15% FBS (Thermo Scientific, Rockford, IL). The obtained osteoblasts from the second digest were allowed to attach to the cell culture dish for 48 h before plating (37°C; 5% $CO_2$).

**Mouse SRC-1 genotyping.**

Offspring of heterozygous matings were genotyped by detection of the disrupted allele in genomic DNA prepared from tail biopsies by enzymatic digestion. Multiplex PCR analysis was performed with four primers (table 7). Cycling conditions were 94ºC for 3 min; 94ºC for 1 min, 57ºC for 1 min, 72ºC for 1 min, 35 cycles; 72ºC for 5 min. The size of the WT PCR fragment was 309 bp and the SRC-1 deficient mouse band was 687 bp (figureS2).

| **Primer** (obtained from Sigma Genosys, St. Louis, MO) | **Primer sequence forward** (provided by Dr. J. XU, BCM, Houston, TX) | **Primer sequence reverse** (provided by Dr. J. XU, BCM, Houston, TX) |
|---|---|---|
| SRC-1 WT | 5'-caaccagcaaaggctgagtcca-3' | 5'-agtacctcctgaggggttagag-3' |
| SRC-1 ko | 5'-tgccgacgcgctagacgatttc-3' | 5'-acacagcaaagaactggaggtg-3' |

**Table 7: Primer sequences for genotyping.**

**MTS assay.**

Cells were plated in a 96-well plate and treated with estrogen (Sigma-Aldrich, St. Louis, MO) or tamoxifen (Sigma-Aldrich, St. Louis, MO) for the indicated timepoints. 20 µl CellTiter 96® Aqueous One Solution Reagent (Promega Corporation, Madison, WI) was added to 100 µl media in each well. The plate was incubated for 4 hours at 37ºC, and the results were obtained by determination of absorbance at 490 nm in a plate reader (BioRad, Philadelphia, PA).

**Actinomycin D treatment.**

Cells were transfected as described above. Actinomycin D (Sigma-Aldrich, St. Louis, MO) (1µg/ ml) was added to each dish 24 h posttransfection. Cells were collected at the indicated timepoints. Total RNA was isolated and q-RT-PCR was performed with the appropriate primers as described above.

**Cycloheximide treatment.**

Cells were transfected as described above. Cycloheximide (Sigma-Aldrich, St. Louis, MO) (20µg/ml) was added to each dish 36 h posttransfection, and cells were harvested at indicated timepoints. Protein lysates were prepared and 100 µg of total protein per sample was analyzed by SDS-PAGE and Western blotting with the appropriate antibody.

**Westernblot.**

Hela cells were washed twice in PBS (Invitrogen, Carlsbad, CA), harvested, and lysed in 5% sodium dodecyl sulfate (Sigma-Aldrich, St. Louis, MO) lysis buffer (100µl/well of a 6-well plate). Extracts were then centrifuged for 5 minutes at 16000g, and the soluble extracts were retained. Samples were normalized for protein content. Cell lysates were separated by SDS-PAGE, transferred to nitrocellulose membrane (Biorad, Hercules, CA), and blocked for one hour in PBS (Invitrogen, Carlsbad, CA) containing 0.1% Tween 20 (Fisher Scientific, Pittsburgh, PA) and 5% milk. The membrane was incubated with the appropriate primary antibody overnight at 4ºC, HRP-conjugated secondary antibody (Amersham, Piscataway, NJ) and analyzed by fluorography (Fluorchem®Q, Alpha Innotech, San Leandro, CA).

**Pulse chase.**

HEK293 cells were were transfected as described above. 24 hours after transfection cells were incubated in DMEM methionine-cysteine-free medium (Invitrogen, Carlsbad, CA) for 1 h and then replaced by medium containing 2mM $^{35}S$ – methionine (PerkinElmer Life And Analytical Sciences, Inc., Waltham, MA) for 40 min. Cells were then washed twice in PBS (Invitrogen, Carlsbad, CA) and incubated with medium containing 2 mM cold methionine. Cells were harvested at the indicated times, and pSG5-flag-SRC-1 was immunoprecipitated from the cell lysates using an anti-flag antibody (Sigma-Aldrich, St. Louis, MO) overnight at 4ºC. Samples were then diluted to 500 µl with lysis buffer, and 30 µl of protein G sepharose (Invitrogen, Carlsbad, CA) to capture the immunocomplex. Samples were incubated 45 min at 4 ˚C with mixing. Beads were washed three times with

lysis buffer, each time spinning 1 min at low speed in a microfuge. Beads were eluted by boiling with SDS sample buffer for 5 min. The immunoprecipates were seperated by SDS-PAGE and bands were quantitated at each time point using phosphor-imaging (Molecular Imager FX, Biorad, Philadelphia, PA).

# 6 Summary

In summary, characterization of genetic variations in ERα coregulators can lead to a better understanding of how a person's individual genetic background influences the development of estrogen-dependent diseases and the efficiency of endocrine treatments. This may enable individual treatments for patients to improve future therapies and diagnostics.

A functional nonsynonymous SNP in the nuclear receptor coactivator SRC-1 that results in an amino acid change from proline to serine (SRC-1 P1272S) was identified. This SNP displays decreased coactivation potential, resulting in decreased activity of the estrogen receptor. The mechanism includes increased protein turnover rate in the presence of the SNP. *In vitro* and *in vivo* data suggest an attenuated response to endogenous and exogenous hormones in bone in the presence of SRC-1 P1272S. Clinical results show that SRC-1 P1272S alters tamoxifen response in bone remodeling. Collectively, our data suggest that carrying this SRC-1 P1272S could result in increased bone loss. Screening for this SNP could potentially predict response of tamoxifen agonistic activity in bone. Presence of the SNP would suggest an individual need for additional bone protective measures. It was shown that identification of genetic variations such as SRC-1 P1272S can contribute to determine individual treatment responses and could be a key feature to personalized medicine in estrogen dependent diseases.

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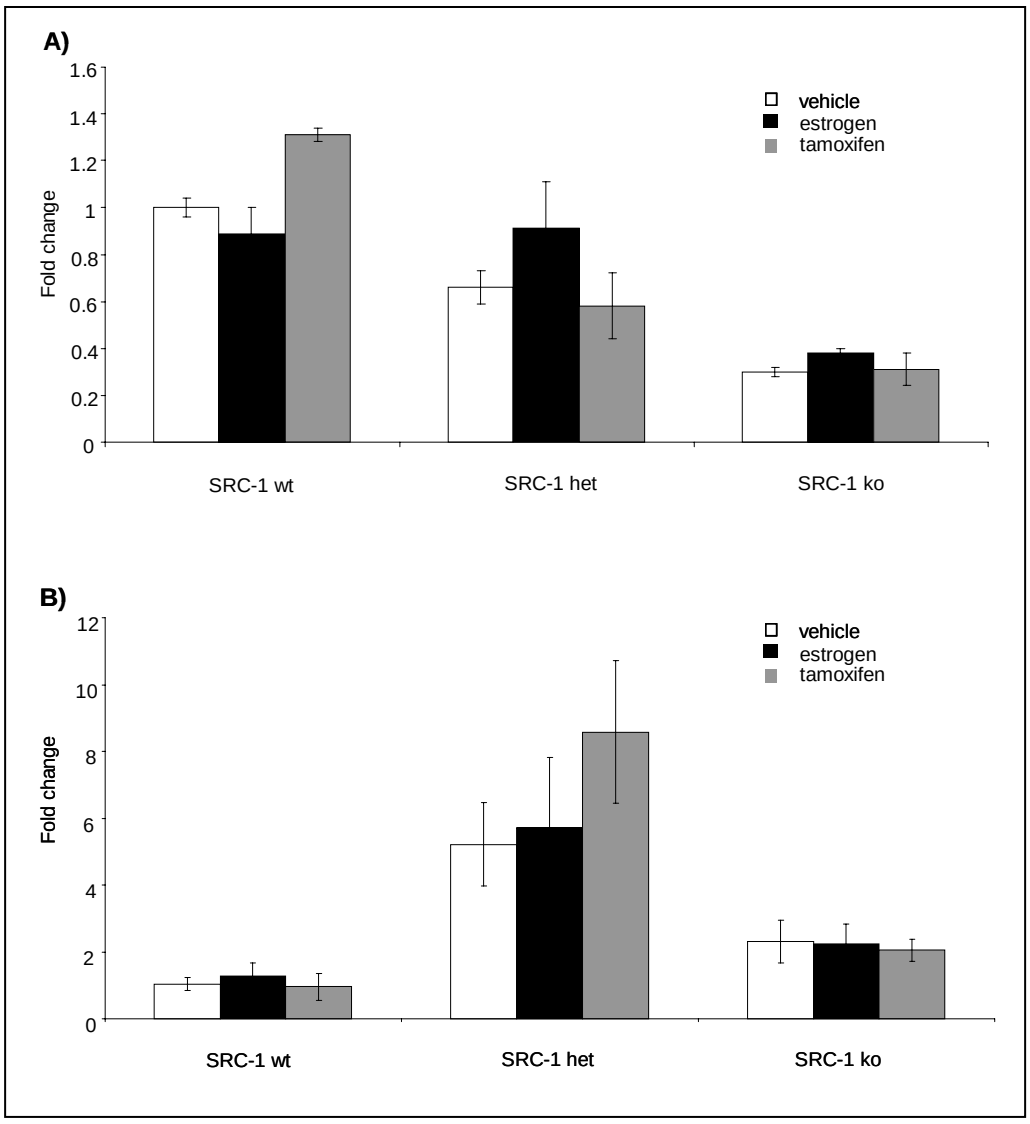
# 8 Appendix


A)
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estrogen
tamoxifen
SRC-1 wt
SRC-1 het
SRC-1 ko
B)
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4
2
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Fold change
vehicle
estrogen
tamoxifen
SRC-1 wt
SRC-1 het
SRC-1 ko

**Figure S 1: SRC-1 osteoblasts express ERα and ERβ.**

**(A) Total RNA was harvested and subjected to qRT PCR using mouse ERα and β-Actin specific primers to determine its expression. Data is expressed as ERα mRNA abundance relative to β-Actin levels. The data is presented as fold over SRC-1 WT relative to vehicle.**

**(B) Total RNA was harvested and subjected to qRT PCR using mouse ERβ and β-Actin specific primers to determine its expression. Data is expressed as ERβ mRNA abundance relative to β-Actin levels. The data is presented as fold over SRC-1 WT relative to vehicle**

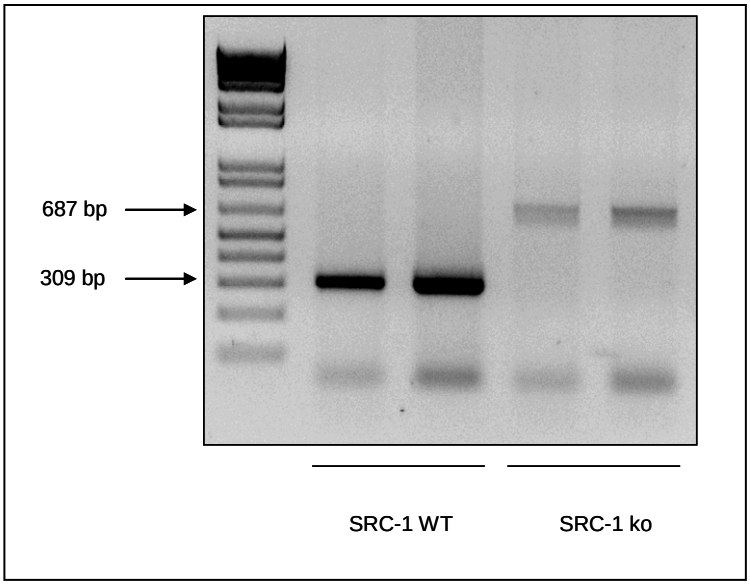


**Figure S 2: Mouse SRC-1 genotyping.**

**Genomic DNA was prepared from tail biopsies. Multiplex PCR analysis for the disrupted SRC-1 allele was performed with four primers (table 6). The SRC-1 WT PCR fragment is 309 bp and the SRC-1 ko band is 687 bp levels.**

# Erklärung

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Halle, 27.12. 2009

Alexandra Richter

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# Danksagung

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Steffi Oesterreich, Ph.D for giving me the opportunity to work and complete my thesis on this interesting topic in her lab

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