Steroid Receptor Coactivator-1 Can Regulate Osteoblastogenesis Independently of Estrogen

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ABSTRACT

Steroid receptor coactivator-1 (SRC-1), a well-studied coactivator of estrogen receptor (ER), is known to play an important and functional role in the development and maintenance of bone tissue. Previous reports suggest SRC-1 maintains bone mineral density primarily through its interaction with ER. Here we demonstrate that SRC-1 can also affect bone development independent of estrogen signaling as ovariectomized *SRC-1* knockout (SRC-1 KO) mouse had decreased bone mineral density. To identify estrogen-independent SRC-1 target genes in osteoblastogenesis, we undertook an integrated analysis utilizing ChIP-Seq and mRNA microarray in transformed osteoblast-like U2OS-ER α cells. We identified critical osteoblast differentiation genes regulated by SRC-1, but not by estrogen including alkaline phosphatase and osteocalcin. *Ex vivo* primary culture of osteoblasts from *SRC-1* wild-type and KO mice confirmed the role of SRC-1 in osteoblastogenesis, associated with altered ALPL levels. Together, these data indicate that SRC-1 can impact osteoblast function in an ER-independent manner.

INTRODUCTION

Bone is an integral and highly specialized tissue that maintains a role in many important bodily functions such as structural support, protection of other organs [1], mineral storage [2], endocrine response [3-5], and hematopoiesis [6, 7]. The strength and structural integrity of bone is maintained by a dynamic process involving the two key components of active bone resorption by osteoclasts and construction of new bone by osteoblasts [8-10].

The estrogen receptor α (ER α) is a major transcription factor involved in female-specific organ development and function [11]. Its role in breast cancer is well studied with the development of selective estrogen receptor modulators (SERMs) like tamoxifen or raloxifene and is one of the best examples for the effectiveness of targeted therapies [12]. However, ER α is also critically important in estrogen (E2) signaling in non-sex-organs such as bone [13]. The importance of estrogen signaling in bone is best represented by (i) the high prevalence of osteopenia/osteoporosis in post-menopausal women [4] and (ii) the success of estrogen replacement therapy and SERMs in preventing post-menopausal bone mineral density (BMD) loss [14]. ER α regulates a nearly unique set of genes in the U2OS osteoblast-like osteosarcoma cell line compared to breast cancer cells [15] and it is hypothesized that tissue specific cohorts of coregulators are critical in determining the downstream targets of ER α [16].

ER α coregulators are proteins that bind to and modulate the activity of ER α [17, 18]. Steroid receptor coactivator-1 (SRC-1), also known as nuclear receptor coactivator-1 (NCOA1), is a coregulator of ER α [19], and has been shown to increase transcriptional activity of ER α in many tissues, including bone [20]. In addition to ER α , SRC-1 has been documented to enhance transcriptional activity for progesterone receptor, glucocorticoid receptor, thyroid hormone receptor, and retinoid x receptor [21]. SRC-1 can also determine whether tamoxifen acts as an agonist or antagonist [16]. Clinically, this translates to high SRC-1 levels associating with decreased disease free survival after tamoxifen therapy [16]. Interestingly, SRC-1 null mouse models revealed that SRC-1 is critical in the hormone response in the uterus, prostate, and

bone [22, 23]. With respect to bone development, loss of SRC-1 can result in osteopenia with high bone turnover in trabecular bone under physiological conditions [24] and skeletal resistance to estradiol in the trabecular but not cortical bone of ovariectomized mice [25]. Our lab and others have reported SRC-1 influences estrogen response and is associated with bone mineral density and tamoxifen resistance in breast cancer [22, 24, 26]. In particular, SRC-1 protects against osteopenia and osteoporosis [24]. SRC-1 has also been demonstrated to act independently of ER. In particular, ADAM22, a non-protease member of the ADAM family of disintegrins, was found to be an ER-independent target of SRC-1 [27]. Thus, SRC-1 plays a major role in tissue specific estrogen signaling, particularly in the bone. Despite this, the molecular mechanisms underlying the role of SRC-1 in bone remain unknown. Critical questions remain unanswered such as what are the SRC-1 dependent target genes in bone and if SRC-1 can act independent from ER α to mediate bone development and maintenance.

Understanding the mechanism of SRC-1 dependent and independent E2 signaling in bone will provide a better understanding of the biology underlying osteoporosis. Thus, we undertook an effort to integrate the ER α & SRC-1 cistromes with the E2 and SRC-1 regulated transcriptomes to identify high confidence E2-independent and E2-dependent, SRC-1 target genes involved in important processes in bone. From our *SRC-1* KO mouse model, we find that SRC-1 regulates a number of genes critical in osteoblast differentiation independent of ER α furthering our understanding of the complex and multifaceted role of SRC-1 in bone development and maintenance.

MATERIALS AND METHODS

Treatment of SRC-1 Knockout (KO) Mice

Generation of the *SRC-1* KO mice has been previously described [22, 25]. Animals were housed in a temperature-controlled room with a daily 12-hour light/12-hour dark schedule and fed a standard laboratory chow diet (Laboratory Rodent Diet 5001; PMI Feeds, Richmond, VA). Pups were genotyped at 21-28 days of age by PCR as previously described [22]. All procedures for these mice were approved by the Institutional Animal Care and Use Committee. *SRC-1* KO and wild-type (WT) litter-mates were divided into sham operated or ovariectomized with supplementation of slow-release placebo or E2 pellets delivering 25 µg/60-day E2 pellet (Innovative Research of America), based on an average body weight of 25 g (n=7-14 per group). After 60 days of treatment, mice were euthanized and specific tissues harvested. The uterus was excised and weighed and the lumbar spines (L1-L4) were harvested and bone mineral density (BMD) analyzed.

Bone densitometry

BMD from the cancellous bone of lumbar (L1-L4) was assessed utilizing microcomputed tomography (µCT) using the 40 Micro CT scanner after 60 days of respective treatments. Endosteal envelope regions of interest were defined on the vertebral bodies of L1-L4 vertebrae starting 100µm away from the vertebral endplates. Bone was segmented from the background using a global threshold of .3g/c. The majority of samples were scanned at 16 micron resolution. Due to technical limitations, a subset of samples was scanned at 10.5 micron resolution. This subset was distributed across all treatment and genotype groups and final BMD measurements were not significantly different due to this technical modification (data not shown).

Chromatin Immunoprecipitation-Sequencing studies with ERα and SRC-1

Chromatin immunoprecipitation assays were performed as described before [28, 29]. Briefly, U2OS-ERα cells (supplied by TC Spelsberg, Mayo Clinic), maintained as described previously [30], were plated at 3.0

x 10⁶ in a 15 cm dish and incubated with 10 nM E2 or vehicle control for 45 minutes. Supernatant was collected and split into ER, SRC-1, and IgG fractions and antibodies specific for ER α (Santa Cruz, HC-20) and SRC-1 (Santa Cruz: M341) added and incubated overnight at 4°C while rotating. Protein G-sepharose beads were incubated with samples for 1 hour at 4°C while rotating. Agarose beads were pelleted by centrifugation at 1000xg for 1 min. Beads were washed at room temperature 5X with wash buffer and eluted with 1% SDS, 100mM NaHCO₃ then heated to 65°C for 4 hours with periodic vortexing. DNA was then purified with the QIAquick Spin Kit (Qiagen). 100 ng of input DNA were used for library preparation and samples were sequenced via the Illumina Genome Analyzer. Reads were aligned to hg18 human genome with ELAND software with default parameters. ER and SRC-1 peaks were called using MACS [31] using p-value cut-off 10⁻⁸ and 10⁻⁵ respectively. ChIPpeakAnno [32] was used to associate each peak to its nearest gene in the human genome. ChIP-seq data is accessible through GEO (GSE26110).

Microarray Platform and Analysis

U2OS-ER α cells (supplied by TC Spelsberg, Mayo Clinic) (2 × 10⁵) were cultured in 6-well plates in appropriate culture medium. The cells were then transfected with 50 nM siRNA using Lipofectamine2000 (Invitrogen). The medium was switched to IMEM + 5% CSS the next day and the cells were treated with vehicle (EtOH) or estrogen (E2) for 3 hr. The following siRNAs were purchased from Dharmacon: ON-TARGETplus Non-Targeting pool (D-001810-10), ON-TARGETplus GSK3 β SMARTpool (L-003010-00), and ON-TARGETplus SRC-1/NCOA1 SMARTpool (L-005196-00). RNA was isolated using Qiagen RNeasy kit and run on an Affymetrix Human Gene 1.0 ST array. Affymetrix CEL files of the microarray experiments were processed using the “affy” package and differential expression was assessed using the “limma” package [33] of the Bioconductor Suite (<http://www.bioconductor.org/>). The threshold for detecting differential expression was set at p-value < 0.01 and q-value < 0.05. Microarray data is accessible

through GEOArchives (GSE90548). Ingenuity Pathway Analysis (Qiagen) was utilized to analyze the functional pathways altered via SRC-1 regulation.

Bone marrow isolation and osteoblast differentiation and alkaline phosphatase assay

Osteoblasts were isolated and differentiated from the long bones of adult mice. Animals were euthanized at 6-8 weeks of age with inhalation of CO₂. After euthanization, mice were rinsed in 70% EtOH and placed in a petri dish. Limbs were removed and cut in half at the joint. Skin and soft tissue were removed from bones with a razor blade and then ends were cut off of each bone. Individual bones were placed inside a 0.6 ml tube with a hole in the bottom, which was placed inside a 1.5 ml collection tube. Bones were centrifuged at 13,000xg for 30 seconds in order to pellet the bone marrow in the larger collection tube. The bone marrow pellets were resuspended in 1 ml cold PBS and subsequently strained through a 70 µm (micron) cell strainer in to a tube containing 10 mls of ascorbic acid-free α-MEM (Gibco, Life Technologies). Growth and differentiation of the osteoblast cultures were done as previously described [34] with some modifications. Cells were pelleted and plated in a T-75 flask, followed by culturing for 1 week with media changes every 2-3 days until cells reached near confluency. Cells were then scraped and reseeded at a density of 5×10^4 per well in a 12-well plate. Osteoblast differentiation was induced in these confluent cells using α-MEM with 10% fetal bovine serum, 50 µg/ml ascorbic acid (Sigma), and 2 mM 2-glycerol phosphate (Sigma). Media were replaced every 2-3 days for 2 weeks. Osteoblast differentiation was qualitatively assessed with alkaline phosphatase staining kit (86R-1KT, Sigma). Whole-field images were taken of wells at 10X magnification on the Olympus Stereoscope SZX16. Quantification of images (n=3 per sample) was then performed in Image J (NIH) and results averaged.

qRT-PCR study of target genes in ex vivo cultures

qRT-PCR was performed using the BioRad CFX 384 machine. Mouse osteoblasts were isolated and differentiated as described above. RNA was extracted (GE Illustra) from cells after 5 and 10 days of treatment with 50 µg/ml ascorbic acid (Sigma), and 10 mM 2-glycerol phosphate (Sigma). 1 µg of total

RNA per sample was used to synthesize the first strand cDNA using iScript reagent (Biorad) in a total volume of 20 μ l. Amplification of triplicate cDNA template samples for the target genes and their primers were performed with denaturation for 15 min at 95°C, followed by 45 cycles of denaturation at 94°C for 15 sec, annealing at 55°C for 30 sec, and extension at 72°C for 30 sec. Values were normalized to Hypoxanthine Phosphoribosyltransferase 1 (HPRT) mRNA. PCR analysis was performed on each cDNA in triplicate and each condition represents the average from RNA isolated from at least 3 mice. All primers were supplied by IDTDNA (Suppl Table 1).

Statistical Analyses

Error bars represent SEM based on the average of the technical replicates from each mouse as referenced in the figure legends. The baseline comparison between the *SRC-1* WT and KO mice was determined by Student's *t*-test, * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$ with appropriate post-test corrections as stated in figure legends. 2-way ANOVA with Sidak's multiple comparisons test was utilized to determine significant differences between vehicle (Veh) and E2 treatments in both non-targeting (NT) and siSRC-1 groups.

RESULTS

SRC-1 is required for normal bone mineral density

Previous studies have published on the loss of SRC-1 on BMD in two different knock-out animal models [23-25]. We utilized the same mouse model as previously published by Modder et al [23, 25], but with two critical modifications: (1) we performed ovariectomy at 8 weeks of age before bones are finished fully developing and (2) utilized 25 µg/60-day slow release E2 pellets as published previously by Yamada et al. [24]. Intriguingly, we observed a decrease in bone mineral density in the lumbar without modulation of hormone levels (i.e. sham ovariectomy) (Figure 1A). Further, after adjustment for this difference in baseline BMD, WT and KO mice responded identically to E2 (Figure 1B). In agreement with previous reports, no difference was seen in uterine weight at baseline [16] (Figure 1C), indicating that the unexpected BMD result was not due to a technical error. Given the effect of SRC-1 on bone in the absence of estrogen, we set out to identify ER-independent targets of SRC-1 in bone cells.

SRC-1 regulates critical osteoblast genes independent of estrogen

We utilized the previously established U2OS-ERα osteoblast-like cell line model [15, 30] to identify SRC-1 target genes in the absence and presence of E2. To identify direct SRC-1 target genes, we used an approach combining genome-wide ChIP-Seq and gene expression studies.

We first identified genomic regions occupied by SRC-1 and ERα by utilizing ChIP-Seq. We observed extensive ERα binding across the genome. In total, 63,686 ERα binding sites were identified (Figure 2A). A majority of the ERα binding sites previously reported in a dox inducible ERα U2OS cell line [15] and many of the ERα binding sites reported in MCF-7 were identified [35] (Supplemental figure 1). We also identified 6,007 SRC-1 binding sites and as expected, the majority of these sites overlapped with ERα binding sites (Figure 2A), confirming that ERα is a dominant nuclear receptor partner for SRC-1 in this cell line model. However, ~40% of SRC-1 binding sites did not overlap with ERα binding sites, suggesting that SRC-1 can be recruited to binding sites independent of ERα.

Gene expression array analysis identified genes regulated by estrogen and/or SRC-1. Overlap of data from ChIP-seq and gene expression array allowed identification of direct targets, and two examples are shown in Supplementary Fig 2 (ISG20 and C3) (Supplemental Figure 2). Interestingly, and in agreement with the finding of ER α -independent recruitment of SRC-1 to genomic binding sites, SRC-1 regulated genes were largely distinct from E2 regulated genes in U2OS-ER α cells (Figure 2B). This indicates that although SRC-1 directly binds to many genomic regions in conjunction with ER α , SRC-1 does not appear to be required for E2 regulation of these target genes. Instead, some of the transcriptional regulatory potential of SRC-1 in U2OS-ER α cells appears to be independent of E2, thus corroborating our *in vivo* results. Thus, in order to identify direct gene regulation of SRC-1 independent of ER, we next performed expression array analysis after targeting SRC-1 levels with siRNA and treating with E2 (Figure 2C). Many regulated genes are involved in ossification and bone development including well-known osteoblast differentiation markers, such as TNFSF11B, ALPL, LIF, and HMOX1 (Figure 2D). In particular, ALPL is a common clinical marker for assessment of bone metabolism and formation [36]. Three out of four SRC-1-regulated genes shown in Fig 2D show significant estrogen response (TNFSF11B, LIF, HMOX1); however, this estrogen regulation is only significantly affected for one of those genes (HMOX1) (two-way ANOVA). For the other genes, the effect of SRC-1 is seen on baseline expression (i.e. in the absence of ligand). Importantly, we identified many SRC-1-target genes that were not regulated by estrogen, yet were significantly altered after SRC-1 knockdown; the top 25 induced and repressed genes are shown in Fig 2E. Many of those genes play important roles in normal bone biology and pathophysiology (highlighted in Fig 2E, on the right). To study the effect of SRC-1 on bone biology in more detail, we next examined primary osteoblast cultures from *SRC-1* KO and WT mice for differences in osteoblast function.

Ex vivo data demonstrates that loss of SRC-1 decreases differentiation of osteoblasts

In order to understand how loss of SRC-1 affects the levels of bone mineral density under normal physiological conditions, we harvested bone marrow from both *SRC-1* WT mice and KO mice at baseline

conditions. As expected, SRC-1 levels are clearly absent in the *SRC-1* KO mouse at both days 5 and 10. Both SRC-2 and SRC-3 levels showed a trend of increased expression in the *SRC-1* KO mice compared to WT but this was not significant (Supplemental Figure 3). This may indicate some low level compensation of the other SRCs for SRC-1, which has previously been demonstrated in cancer [37]. Given the identification of osteoblast differentiation genes in our gene expression analysis, we focused our studies on osteoblast phenotypes. Specifically, we identified genes regulated by SRC-1 and not E2 and ER α ($p < 0.05$) that are known to play important roles in the growth of bone tissue, size of bone, and function of differentiation and proliferation of osteoblasts via Ingenuity Pathway Analysis (Supplemental Table 2). Alkaline phosphatase (ALPL) staining is a classical assay to test for differences in osteoblast differentiation potential and an important determinant of bone formation as mentioned previously. *Ex vivo* osteoblast differentiation assay revealed *SRC-1* KO bone marrow cells produce significantly less ALPL staining compared to WT bone marrow cells (Figure 3A-3B), thus this indicates that osteoblast function in *SRC-1* KO mice could be impaired through a reduction in osteoblast differentiation potential. To further confirm these findings, we examined the expression of genes critical for osteoblast differentiation. A strong but non-significant decrease was observed in ALPL expression levels further supporting the ALPL staining results (Figure 3C) while Osteocalcin OCN was found to be significantly decreased in *SRC-1* KO bone marrow cells after 5 days of differentiation (Figure 3D).

DISCUSSION

Our study indicates that the BMD of *SRC-1* KO mice are lower in baseline conditions but the response of these KO mice to E2 was similar to WT mice. This is in contrast to previous reports [24, 25]. Potentially

these differences are a result of variables that differed between the three studies, particularly age at ovariectomy and E2 dose. Similar to Yamada and colleagues [24], we found that loss of SRC-1 resulted in high bone turnover and osteopenia under normal physiological conditions and that response to E2 supplementation was extremely impaired. Modder and colleagues [25] did not observe any effect at baseline with loss of SRC-1, however, they did obtain a minimal response to E2 in ovariectomized *SRC-1* KO mice. Our dose of E2 was similar to that used in Yamada and colleagues, while the age of our mice at 8-weeks was the youngest out of all three studies. We chose to use the mice at 8-weeks of age because this is the time that C57bl/6 mice reach sexual maturity and the growth of bones begins to slow [38]. As a result, we believe this age provides the best baseline before endogenous systemic estrogens rise and that this effect is lost at later time points. However, since we did not measure estradiol levels in the mice used in our study, thus we cannot fully exclude the possibility of some remaining low levels of estradiol that might activate ER.

Nevertheless, our ChIP-Seq and expression array profiling revealed numerous SRC-1 regulated genes that were not dependent on E2 signaling, providing further evidence for E2-independent action of SRC-1. This included classic osteoblast regulatory genes such as ALPL and osteocalcin (OCN). Importantly, although we were able to identify previously known ER α binding sites and E2 regulated genes, genes most critical for osteoblast differentiation and function were largely independent of ER α and E2. Further analysis of these SRC-1 regulated genes in Ingenuity Pathway Analysis also revealed those important in connective tissue development under the biological functions pathway, which included important functions such as size of bone (ALPL, CAV1, CD40, LIF), differentiation of bone (JAG1, NOTCH2, CSF2, SPP1) and proliferation of osteoblasts (TNFRSF11B, TGFBR1/2, IGF1R). This could account for the loss of BMD observed in both the sham-treated and ovariectomized mice for the *SRC-1* KO genotype compared to *SRC-1* WT mice, as there is a lack of functional osteoblasts to form bone. This was further supported by *ex vivo* differentiation and gene expression studies. Together, these data demonstrate that SRC-1 has a major role on bone development independent of E2 signaling.

OCN is produced solely by osteoblasts and assists in the mineralization process of bone by binding to hydroxyapatite, thus it is often used as a biomarker for bone formation [39]. Therefore, we demonstrate that loss of SRC-1 clearly affects bone formation rates by altering the ability of the osteoblasts to secrete ALPL and OCN, which are two key factors in osteoblastogenesis. Although it is not currently clear which nuclear receptor(s) that SRC-1 is acting through, it is clear that SRC-1 is a critical regulator in bone development. Several studies in the literature demonstrate that Vitamin D receptor (VDR) readily recruits SRC-1 to bind to the OCN promoter after stimulation with Vitamin D3, in order to enhance bone specific gene transcription [40-42]. PPAR γ has also been shown to suppress osteoblastogenesis and activates osteoclastogenesis, thereby decreasing bone formation while sustaining or increasing bone resorption [43]. SRC-1 is a known coactivator of PPAR γ in the adipose tissue [44]. Interestingly, our array studies revealed altered expression of PPAR γ upon SRC1 knockdown (data not shown), and thus it would be of interest to determine whether a PPAR γ -SRC-1 complex plays a role in bone biology. Clearly, additional studies are necessary to fully understand ER-dependent and ER-independent role of SRC-1 in normal bone biology and pathophysiology.

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325 Histomorphometry Core.

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FIGURE LEGENDS

Figure 1: SRC-1 is required for estrogen-independent maintenance of BMD. WT and KO mice underwent sham surgery or ovariectomy followed by placebo or estrogen pellet supplement for 60 days. BMD from the cancellous bone of the lumbar was measured by Micro CT analysis. Number of mice per group was 6-9 for wild-type and 10-13 for KO. (A) Effect of SRC-1 and estrogen on BMD shows a significant decrease in BMD between WT and KO mice. *, represents $p < 0.05$ by unpaired student's *t*-test and corrected with multiple comparisons using an FDR cut-off of 1%. (B) After normalizing for baseline effect, no effect of SRC-1 on E2 dependent BMD was observed. T-test was conducted to the respective sham controls. ***, represents $p < 0.0005$ performed by two-way ANOVA corrected for multiple comparisons using a Bonferroni post-test. (C) Uterine weight was examined as a positive control showing no baseline difference and a reduction in estrogen response in KO mice in agreement with previous findings (Xu J et al., Science 1998). *, represents $p < 0.05$ by unpaired student's *t*-test

Figure 2: Identification of estrogen independent SRC-1 target genes.

U2OS-ER- α cells were transfected with either non-targeting siRNA or siRNA targeting SRC-1, which were then treated for 3 hours with either vehicle control (ethanol) or E2 (n=3 replicates per treatment group) (A) ChIP-seq was performed and 6,007 SRC-1 binding sites were identified and the majority of these sites overlapped with ER α binding sites. (B) SRC-1 regulated genes were largely distinct from E2 regulated genes in U2OS-ER α cells. (C) Microarray was performed on the Human 1.0 ST gene array (Affymetrix). A heat map demonstrates E2 and SRC-1 regulated genes (n=1600) with some genes of relevance to bone biology highlighted. (D) Gene expression profiles for highlighted genes as examples of SRC-1 regulation are graphed demonstrating the fold change with respect to non-targeting vehicle control. Error bars represent the standard deviation. Test for significance was performed using t-test and 2-way ANOVA, for effect of estrogen and for interaction between estrogen and SRC-1 knockdown, respectively. (E) 25 top

genes that i) were significantly induced or repressed genes upon SRC-1-knockdown, and ii) did not show significant effect by estrogen.

Figure 3: SRC-1 WT mice demonstrate increased alkaline phosphatase staining compared to SRC-1 KO mice. Bone marrow was harvested from both WT C57Bl/6J (n=6) and SRC-1 KO mice (n=5). Cells were differentiated with 50 µg/ml ascorbic acid (Sigma), and 2 mM 2-glycerol phosphate (Sigma) for 2 weeks and then stained for ALPL production. (A) Experiments were performed in triplicate for each mouse with representative images for each shown. (B) Quantification of intensity was performed with Image J software (NIH). Student's unpaired *t*-test, **p*<0.05. qRT-PCR analysis demonstrates that loss of SRC-1 results in decreased OCN expression after 5 days of differentiation. Bone marrow was harvested and cells were differentiated with 50 µg/ml ascorbic acid (Sigma), and 2 mM 2-glycerol phosphate for 5 days and then RNA collected and converted into cDNA for expression analyses. Expression levels of (C) ALPL and (D) OCN genes were analyzed in both WT C57Bl/6J (n=6 replicate assays) and SRC-1 KO mice (n=12 replicate assays) after 5 days of differentiation. **P*<0.05, ****P*<0.0005, Student's *t*-test.

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Figure 1

Figure 2

Figure 3

A

B

C

D