CHARACTERIZATION OF ESRRB FUNCTION IN METASTATIC PROSTATE CANCER CELLS AND TRANSCRIPTIONAL REGULATION OF HEDGEHOG-SIGNALING PATHWAY TARGET GENES

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The undersigned, appointed by the dean of the Graduate School, have examined the dissertation entitled

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CHARACTERIZATION OF ESRRB FUNCTION IN METASTATIC PROSTATE CANCER CELLS AND TRANSCRIPTIONAL REGULATION OF HEDGEHOG-SIGNALING TRANSDUCTION PATHWAY TARGET GENES

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ABSTRACT

Orphan nuclear receptor Estrogen Receptor Related Receptor β (Esrrb) is a transcription factor. It is intensively studied for its function in embryo development and induced pluripotent stem cell induction. Although it was also shown to be important in cancer, little is known about its function in cancer cells and cancer relevant pathways. In this dissertation, we focus on Esrrb's transcription targets discovery in prostate cancer cells, as well as its function in regulating Hedgehog (Hh)-signaling and Akt signaling pathways.

Here we report our discovery of Esrrb-targeted genes in metastatic prostate cancer cells and distinguish a group of target genes responsive to the Esrrb selective ligand DY131. Although there is argument about whether the intrinsic transactivation activity is ligand dependent, we found Esrrb has both ligand-dependent and ligand-independent transactivation activities. We also characterized a collection of Esrrb- (67 genes) and Esrrb-dependent DY131- responsive (1161 genes) genes and defined a group of genes for which DY131 serves as an agonist or antagonist through Esrrb. These results expand

the understanding of the transcription regulatory function of Esrrb and provide a handful of reference markers regarding Esrrb activity. To understand the mechanism of Esrrb-driven gene expression alteration, we built a bioinformatics pipeline that predicts transcription factor binding and target genes using an in-house bioinformatics tool. The comparison between the DY131-activated Esrrb-driven gene regulation network and the ligand independent Esrrb gene regulation network infers that DY131 expands the effects of Esrrb. Gene set enrichment analysis shows Esrrb target genes are related to cell proliferation, regulation of apoptosis and transcription regulation, supporting its role as a transcription factor and its known function in inhibiting prostate cancer cells proliferation.

Esrrb, as well as the Hh-signaling pathway, are known for their functions in inducing pluripotent stem cells and their important role in tumorigenesis. We hypothesize that the Esrrb and Hh-signaling pathways functionally overlap in gene expression regulation, and that Esrrb can regulate Hh-signaling activity. Using RNA-Seq in an Esrrb-expressing Hh-responsive cell line and an in-house innovative computational decision tree gene-sorting tool, we sorted Hh-signaling and Esrrb-responsive genes in different groups based on their mRNA concentration in different conditions. In addition to a full list of Hh-signaling and Esrrb target genes, we found 109 Hh-signaling differentially responsive genes, which respond to Hh ligand differently with or without Esrrb expression. The presence of these genes clearly show that Esrrb is capable of regulating Hh-signaling pathway activity. In addition,

co-treatment of DY131 with Hh ligand completely removed Hh-ligand's effect on all tested Hh-target genes without Esrrb expression, supporting the reported direct SMO inhibitory role of DY131. However, when Esrrb is expressed, DY131 treatment lost the anti-Hh effect on a group of genes including Sfrp2, Prl2c3, Hp, Hoxd8, Dpt, Pdcd4, Smoc2, Hsd11b1 and Ogn, indicating DY131 also regulates these genes in an Esrrb-dependent manner and Esrrb ligand can be used to alter these genes' response to Hh-signaling activation.

Since Akt activity was reported to be responsive to Hh-activated Smoothened (SMO), and Akt over-activation was reported to be one of the mechanisms of anti-Hh treatment Vismodegib resistance, with the result that DY131 is an SMO inhibitor, we hypothesized DY131 can inhibit SMO-driven Akt activity. We found Akt phosphorylation (pAkt) is stimulated by Hh treatment, and the addition of DY131 can inhibit both basal level and Hh-stimulated pAkt. A similar pAkt inhibition is also observed in DU145 cells, which are SMO inactive and Esrrb null, indicating the pAkt inhibition is not SMO or Esrrb dependent. Interestingly, although DY131 is likely to inhibit pAkt by binding to Esrrb, Esrrb itself also has the ability to inhibit pAkt in DU145 cells. These results strongly indicate either DY131 or Esrrb can be used to prevent Vismodegib resistance.

Overall, our comprehensive analysis of Esrrb-regulated gene expression shows that Esrrb is a significant factor regulating cellular proliferation and apoptosis. Its

activity in regulating Hh-signaling target gene expression and Akt inhibitory effect indicates Esrrb can potentially serve as a therapeutic target in cancer treatment.

CHAPTER I

LITERATURE REVIEW

This dissertation focuses on the discovery of gene expression regulatory function of Estrogen Receptor Related Receptor β (Esrrb), Esrrb targeted genes, as well as Esrrb interactions with the Hedgehog (Hh)-signaling pathway. Esrrb belongs to the nuclear receptor superfamily, and shares sequence similarity, co-regulatory proteins, some man-made ligands with Estrogen Receptors. In the following literature review, a brief description of Esrrb discovery and molecule structure will be introduced, followed by a summary of Esrrb ligands. Known function of Esrrb will be discussed in detail. Finally, Hh-signaling pathway components, target genes and function will be described.

PART I: ESTROGEN RELATED RECEPTORS (ESRRB)

Discovery

Human ERRβ cDNA was first isolated from a cardiac cDNA library using Estrogen Receptor DNA binding domain as the probe by reduced stringency hybridization [82]. Following studies using an expressed sequence tag (EST) database to find novel nuclear receptors revealed that the original cloned "human" Esrrb was actually rat Esrrb. The real human Esrrb was named human ERRβ2 [33]. Hereinafter, all human Esrrb sequence refers to human ERRβ2 sequence. Mouse Esrrb was isolated and cloned 8 years later from mouse embryonic stem cells and embryonic carcinoma cells cDNA library by RT-PCR using degenerated primers complementary to P-box and Ti-domain sequences of nuclear receptor [183].

Molecular structure and splicing isoform

The Lubahn lab found that human Esrrb had three splicing isoforms: 1) Full length hEsrrb (hERRβ2) has all 12 exons; 2) hERRβ2-Δ10, which lacks exons 10 and

12; and 3) short-form hEsrrb (SFhERRβ), which lacks exons 10-12 and uses intron sequence to form a distinct carboxyl-terminal for the short form receptor (Figure I-1) [284]. Like other nuclear receptors, Esrrb has: 1) a ligand independent Activation Function 1 (AF1) domain; 2) a DNA binding domain, which recognizes the half site of the estrogen response element, also known as the Estrogen Related Receptor Response Element (ERRE) or Steroid Factor Response Element (SFRE); 3) a linker domain, and 4) a ligand dependent AF2 domain, which binds to ligands and co-activators/ co-repressors. hERRβ2 and hERRβ2-Δ10, but not SFhERRβ, have a C terminal F domain. The lack of exon 10 in the hERRβ-Δ10 isoform causes the open reading frame to be shifted, and thus the sequence homology of the C terminal domains of the two isoforms is very low [284].

Early studies on Esrrb show that it has very limited expression in adult organs with the only organs with positive expression being the testis, kidney, cochlea, and retina [46, 82, 183, 284]. The newly discovered hERRβ2-Δ10 and SFhERRβ showed distinct patterns of expression. Similar to hERRβ, hERRβ2-Δ10 was only found in testis, kidney, cochlea and retina. In contrast to the two longer Esrrb splicing isoforms, SFhERRβ has a much broader range of expression among the organs tested, including skin, testis, breast, kidney, placenta, prostate, ovary, cervix, skeletal muscle, adult and fetal heart, adult and fetal brain, spleen, salivary gland, stomach, lung, thymus, trachea, small intestine, spinal cord, cerebellum, cochlea, retina and colon, but not in uterus. SFhERRβ shares high homology with mouse and rat Esrrb and it is the real human ortholog of mouse and rat Esrrb. In species other than human, similar splicing isoform

was not found [46, 284]. All three human Esrrb splicing isoforms have similar effects in published results, but the difference in C terminal domain affects the potency of the Esrrb function, as summarized in the later part of this review.

Esrrb ligand

Esrrb is one of the orphan nuclear receptors and it is believed to have constitutive transcriptional regulation activity. One hypothesis explaining Esrrb endogenous activity suggests that the LBD of Esrrb forms an active conformation. This hypothesis is supported by X-ray crystallography studies with Estrogen Related Receptor Gamma (Esrrg) LBD, which is highly similar to that of Esrrb [31, 252]. On the other hand, the constitutive transcriptional activities of Esrrb are observed only under cultured conditions when un-stripped serum is used and where the inherent activity is absent when cells are cultured in medium supplemented with charcoal-stripped serum. This suggests that there is at least one hydrophobic small molecule that is required for Esrrb to generate its function [246]. Though there is not much known about Esrrb native ligand(s), there are several botanical originated compounds and synthetic compounds that show modulation of the transcriptional activity of Esrrb.

1. DY131/GSK4716:

Since the Glu and Arg in the Estrogen Receptor ligand-binding pocket that form essential hydrogen bonds with the A ring of estradiol are conserved in Esrrb, phenolic compounds were thought to be potential candidates for Esrrb binding. Two phenolic acyl hydrazones compounds are synthetic chemicals capable of modulating Esrrb

activity, which were isolated in a screen of a small phenolic compounds library. GSK4716 and DY131 showed agonist effects similar to Esrrb cofactor PGC1α in Estrogen Response Element (ERE) driven reporter assay in Hela cells transfected with exogenous Esrrb. GSK4716 and DY131 showed similar efficacy as PGC1α at 10uM and was determined as agonists of Esrrb [4]. DY131 was also shown to stimulate hCYP19 expression in syncytiotrophoblasts to inhibit the growth of ERα+ endometrial cancer cells while stimulating the growth of the ERα- cells, and to stimulate the proliferation of brain tumor cells U87MG T98G, as well as hedgehog-signaling inhibition activity [79, 119, 255, 269]. Although both compounds showed promising effects on modulating Esrrb transcriptional activity, GSK4716 and DY131 cannot differentially regulate Esrrb from Esrrg. One reason, as mentioned earlier, is that Esrrb and Esrrg share high homology in their LBDs. However, another problem for these two compounds is the binding affinity to Esrrb, unlike Esrrg, is unknown due to the lack of a known leading compound that bind to Esrrb. Since Esrrb and Esrrg share high homology in their LBDs, we have reason to assume GSK4716 and DY131 bind to Esrrb since both compounds bind to Esrrg with high nM and low uM range in competitive binding assay using [H3]-labeled 4-hydoxy Tamoxifen [286].

2. Biochanin A/Genestein/ Daidzein/6, 3′, 4′-trihydroxyflavone:

Isoflavone Biochanin A, Genestein and Daidzein, flavones 6, 3′,4′-trihydroxyflavone were shown to activate exogenously expressed Esrrb in an ERE-driven luciferase assay performed in Hela cells. At 10uM concentration, biochanin A and 6, 3’, 4’-trihydroxyflavone activates Esrrb by 2 fold while genestein and daidzein

stimulate the receptor 1.5 fold [219]. Unlike DY131 and GSK4716, which activate both Esrrb and Esrrg, genestein and daidzein do not activate Esrrg at 10uM, but all four compounds activate Estrogen Related Receptor Alpha (Esrra), indicating the amino acid sites that are required for flavones and isoflavone are different from phenolic acyl hydrazones. Yeast two-hybrid assay also showed that Biochanin A, Genestein and Daidzein, at 10uM, enhance the binding between Esrrb and cofactor PNRC, while 6, 3′, 4′-trihydroxyflavone show no enhancement [219]. These phytoestrogens showed promising effects on modulating Esrrb effect, but since the Kd of the potential binding to Esrrb is unknown and the concentration tested overlaps with the concentration of these compounds effect on ERs, the phytoestrogen in this group is not a reliable tools when ERs are present [47, 143, 203].

3. Tamoxifene (TAM)/ 4-OH Tamoxifene (4-OH-TAM):

TAM and its metabolite 4-OH-TAM are classic Selective Estrogen Receptor Modulators (SERMs). Both compounds were shown to dissociate cofactors from Esrrb and Esrrg, but not Esrra in FRET and yeast two-hybrid assays [49, 239]. Although there is no direct ligand binding data showing TAM or 4-OH-TAM directly binds to Esrrb, Esrrg was confirmed to bind 4-OH-TAM [87, 133] with low nM binding affinity.

4. Diethylstilbestrol (DES):

The classic endocrine disruptor, DES, was found to deactivate Esrrb, supported by the evidence that DES dissociates GRIP1 from Esrrb by FRET assay with a IC50 of 1uM, and DES decreases Esrrb's activity in the ERE-driven luciferase reporter gene in

COS-1 cells with IC50 ~10uM[240]. 10uM DES also caused SRC1 release from Esrrb [49, 240]. Although there is no direct binding affinity reported, we could assume the binding affinity from DES binding to Esrrb is in the nM range since DES binds to Esrrg with 13nM Kd and DES has almost the same effect on Esrrg in cofactor releasing. What makes DES an important tool for Esrrb study is that DES showed *in vivo* effects similar to genomic knock out of Esrrb, when administered to pregnant mice. Esrrb is essential for placenta formation and knock out of Esrrb in mice led to the formation of abnormal chorion, diploid trophoblast self renewal failure and an increased number of trophoblast giant cells. Treatment of DES to pregnant mice from 4.5 to 8.5 days post coitum (dpc) led to a decrease of the labyrinth and spongiotrophoblast layers and an increase in the giant cell layer, which is similar to the Esrrb null phenotype [137, 240].

Biological Function of Esrrb

1. Embryogenesis:

Esrrb is abundantly expressed in embryonic stem (ES) cells, but once ES cells have been differentiated to endoderm-like cells by exposure to retinoic acid, the mRNA concentration of Esrrb drops sharply to below the detection limit. Esrrb was found to express in amniotic fold cells derived from ectoderm 6.5 dpc. When chorion is formed from the amniotic fold in day 7.5 dpc, Esrrb expression is still high while at 8.5 dpc, when chorio-allantois is fused, Esrrb is not detectable in chorionic plate of early placenta, nor any other tested region in the embryo [183].

Anther group also reported that Esrrb is a marker of diploid extraembryonic ectoderm [231]. The first phenotype that was reported related to Esrrb is the impaired formation of placenta after genetic knock out of Esrrb in mouse, characterized by excessive differentiated trophoblast giant cells and a deficiency of diploid trophoblast stem cells [137]. The question arising from these studies is whether Esrrb is the inhibitor of differentiation or if it goes away along with differentiation. Induced pluripotent stem cell (IPSC) studies tend to suggest that Esrrb is the activator of the dedifferentiation and thus Esrrb pushes the stem cells toward maintaining stemness in the balance between differentiation and self-renewal. Although the mouse Esrrb knockout is embryonic lethal, the placental defect can be rescued by making chimeras of wild type tetraploid and Esrrb knock out embryos. The rescued embryos, once past trophoblast formation, can develop into adults and both male and female mice are fertile without defects in the development of gonad and primordial germ cells. However, in both male and female Esrrb null mice, germ cell numbers are decreased and the mutants have abnormal behavior, including falling when walking and backward and circular movements, indicating Esrrb's role in gonadal germ cell proliferation, inner ear development, inner ear balance control function, and potentially brain function regulation [46, 153, 215].

2. Stem Cell Maintenance and iPS cell Induction:

iPS cells are a good substitute of Embryonic Stem Cells (ESCs) without the associated ethical issues, while still retaining the pluripotency of ESCs. iPSC and ESCs are highly similar in transcriptome, epigenome, proteome and metabolome, making iPSC a very hot research topic for basic stem cell biology, development biology,

regenerative medicine and biotechnology [65, 74, 95, 134, 158, 175]. Yamanaka factors (Sox2, Klf4, C-myc, Oct4) were found to be the core transcriptional factors that have the ability to reprogram differentiated cells into iPSCs. Interestingly, Esrrb was found to have the ability to replace c-myc and klf4 to induce the reprogramming of fibroblast cells to iPSC [70]. Although the efficiency of the Esrrb driven reprogramming is slightly lower than with the Yamanaka factors, partially due to the ability that Esrrb can induce cell cycle arresting by up-regulating p21 [28, 70]. Two back-to-back papers reported that Esrrb binding to the promoter of reprogramming genes is dependent upon Steroid Receptor Cofactor 3 (SRC3) [179, 264]. Esrrb has also been shown to be a target of GSK3-Tcf3 axis in the regulation of embryonic stem cell renewal. Additionally, Esrrb is a target of Nanog and was shown to be capable of replacing Nanog in iPS cells induction [72, 142].

Although Esrrb is viewed as a substitute for some of the Yamanaka factors, a report based on single cell reprogram analysis indicated that Esrrb, along with Utf1, Lin28 and Dppa, are more efficient reprogramming factors [19]. Transcriptome analysis and systematic RNAi screen discovered there are three phases to the reprogramming: 1) initiation, 2) maturation and 3) stabilization. Each is marked by different gene changes and epigenetic features [129, 200]. Esrrb function was shown to be at the very early stage of the reprogramming, and a second late phase of reprogramming involved the activation of Sox2, which is activated by Esrrb, and sequentially the down-stream gene activation that finally lead to stabilized pluripotent state [19, 70].

3. Hearing Impairment:

There are very limited phenotypes associated with Esrrb gene mutation. Beside the embryonic lethal effect from Esrrb knock out study, Autosomal-recessive nonsyndromic hearing impairment (ARNSHI) is one of the major phenotypes in humans that are correlated with Esrrb mutations [9, 35, 46, 125, 137, 199]. Esrrb is expressed in nonsensory epithelia of the vestibulum, in the developing stria vascularis and lateral wall of the cochlea, and in the vestibular ganglion [35, 46]. Four papers reported that mutations in human Esrrb are strongly associated with the human ARNSHI pathological process based on linkage analysis and mutation sequencing. Interestingly, except for one mutation found in the DNA binding domain, all of the other mutations are located in the LBD (Table I-2), indicating that the potential failure of ligand binding or co-factor binding to the receptor is the fundamental reason for the Esrrb-related hearing loss [9, 46, 125, 199].

Although a specific molecular mechanism is lacking, one can imagine that mutated Esrrb harboring a deformed LBD failed to bind to endogenous ligand or interact with co-factors, and thus, considering the iPSC induction ability of Esrrb, fails to renew the progenitor cells in the inner ear. This lack of induction leads to the final destruction of inner ear structure and the aftermath hearing loss.

The circular behavior of the rescued Esrrb knockout mice, and also the conditional knockout mice (*Nr3b2*$^{-/-}$ cochlea), was thought to be a classic symptom of defective vestibular function. The tissue specific knockout of Esrrb in cochlea resulted in the decrease of several ion channel and transporter genes in strial marginal cells and

vestibular dark cells, including Aldh1a2, Rspo3, Ptgds, Atp1b2, Slc12a2, Kcnq1, and Wnk4*,* which are important for inner-ear structure, function, and the homeostasis of inner-ear fluid, revealed another aspect of the pathophysiology of DFNB35 hearing loss [27, 35, 53, 73, 101, 127, 153, 180, 215, 256].

Though the correlation between Esrrb mutations and recessive hearing loss is strong in humans from consanguineous family genetic and sequencing analyses, similar mutations are not found in other species. In the case of mouse, most inbred strains will have hearing loss in the late part of their life, while most outbred strains keep sensitive hearing ability when they are old, reflected by their significantly lower hearing threshold at different sound wave frequencies. Although cdh23 had been found to be a classic gene that once mutated and would result in hearing loss in mouse; we were still trying to find out whether the hearing loss mouse strains harbor any Esrrb mutation compared to wild mouse, and whether Esrrb mutation is a modifier gene that correlates with cdh23 mutation [54]. To our surprise, no mutation was found in Esrrb gene in mouse strains with hearing loss. In human Esrrb, the V342L mutation was found to be associated with hearing loss in a consanguineous family from Turkey, while this amino acid site in mice is not Val but rather a Met, in both inbred and outbred strains, eliminating the possibility that this Met is the reason for the inherited hearing loss in inbred strains.

4. Tumorigenesis and Tumor Progression

Since Esrrb was thought to express only in embryonic tissue but not in most adult tissues, the potential effects on Esrrb in tumorigenesis and cancer development has not

been well studied. As mentioned earlier, the short form splicing isoform of Esrrb is expressed in adult tissue. Immunohistochemistry results showed that Esrrb concentrations are much lower in metastatic prostate tumor cells than in localized prostate cancer cells and normal prostate epithelium cells [274]. Esrrb concentration was shown to be highest in fetal prostate, and the protein concentration decreases as the prostate tumor develops, with the lowest concentration found in advanced prostate cancer [274]. The decrease in expression of Esrrb has been shown to be the result of hypermethylation of Esrrb gene. Re-expression of Esrrb was shown to inhibit prostate cancer cell proliferation through the up-regulation of cyclinD1 inhibitor p21 by directly binding of Esrrb to p21 promoter, and facilitated by RIP140 family transcription factors [28, 274].

Two groups, including our lab, have reported the inhibitory effect of Esrrb on nuclear receptor transcription activity. Esrrb is so far reported to inhibit ERα, ERβ and Glucorticoid receptor (GR). Instead of a general scavenger effect, Esrrb cannot inhibit Progesterone Receptor's activity on the Progesterone Response Element-driven luciferase reporter [238]. Esrrb's transcriptional activity inhibition of ERs is induced by the formation of heterodimer formation between ER and Esrrb at the estrogen response element (Jinghua Liu, unpublished). Although there is no direct evidence that Esrrb binds to the half-site of estrogen response elements as it associate with ERs, co-immunoprecipitation and truncation analysis of Esrrb support the model that Esrrb binds to ER, forming a heterodimer and recruiting co-repressor to inhibit nuclear receptors transcriptional activities. A recent paper from our lab showed that ERβ knock

out TRAMP mice had a significantly higher incidence of advanced prostate cancer compare to ER wild type TRAMP mouse, while ERα knock out TRAMP mouse had a decreased frequency of advanced prostate cancer, indicating that ERα is pro-cancer and ERβ is anti-cancer [209]. We hypothesize that finding a way, through either compound(s) or cofactor(s) that can manipulate the function of Esrrb, and thus to differentially regulating ER activities, will be an efficient way to inhibit prostate cancer growth.

Another function of Esrrb is the inhibitory effect of NF-E2 related factor 2 (Nrf2) transcriptional activities at the Antioxidant Response Element (ARE) [157, 248, 285]. Though Nrf2 inhibition is not a unique function of Esrrb, Esrrb has the most potent inhibitory effect among ERR family nuclear receptors, and estrogen activated ERα. Among Esrrb splicing isoforms, the short form isoform is the most potent transcriptional inhibitor of Nrf2 compared to the other two Esrrb splicing isoforms [4, 285]. Considering Nrf2's key effect in inducing antioxidant proteins and phase II detoxifying enzymes by binding to ARE in the regulatory sequences of genes involved in the Reactive Oxygen Species (ROS) response, Esrrb could be a significant drug-able target of cancer, neurodegenerative disease and atherosclerosis [77, 248, 285].

PART II: HEDGEHOG (Hh) SIGNAL TRANSDUCTION PATHWAY

Pathway Components

Hedgehog (Hh) signaling consists of two membrane proteins, Patched (Ptch), which serves as the receptor of Hh ligand, and Smoothened (SMO), which is the major

switch of the downstream pathway [25, 289]. When Ptch is not bound by Hh ligand, Ptch inhibits SMO translocation into primary cilia by inducing the β-arrestin mediated internalization of SMO, thus preventing physical contact between SMO and downstream signaling molecules [38, 48, 118, 144, 195] (Figure I-3). Beside the physical hindrance that Ptch creates for SMO, Ptch constitutively pumps out steroid-like compounds, such as oxysterols and Vitamin D3. Vitamin D3 has been shown to bind to SMO and lock SMO into an inactive conformation [13]. When Hh ligands bind to Ptch, the conformation change of Ptch eliminates the SMO inhibition effect by allowing SMO to translocate into the tip of the primary cilium mediated by intraflagellar transport (IFT) [144]. In parallel to the above Vitamin D3 mechanism, Ptch can pump oxysterols instead of Vitamin D. Several papers reported that oxysterols, a group of important cholesterol metabolites, could bind to SMO and act as Hh-signaling pathway activator [13, 59, 163, 167, 193].

When SMO is activated after Hh ligand binding to Ptch, the Hh signal is carried along by a series of several intermediate signaling molecules, including Fused (Fu), Suppressor of Fused (Sufu), and finally leading to the cleavage, nuclear translocation and activation of the Gli transcription factors [177, 217]. Gli transcription factors recognize their DNA-binding motif and regulate gene expression, as well as interact with other transcriptional factors and transcription cofactor [40, 83, 117, 130, 250].

Many signaling-transduction pathway components are known to interact with the Hh-signaling pathway, while most of the pathways are shown to work by interacting with Gli transcription factors. These include Protein Kinase A (PKA), Glycogen synthase kinase 3 (GSK3β), Casein Kinase 2 (CK2), Androgen Receptor (AR), Protein Kinase B (Akt), Protein Kinase C (PKC), Extracellular signal-regulated kinases (ERK) and p53, making Gli a core factor incorporating the signals from multiple pathways [34, 107, 168, 170, 191, 214].

Target genes

The Hh-activated Gli transcription factor regulates several classical Hh target genes including: Gli1, Ptch1, Ptch2, Hhip1, MycN, Ccnd1, Ccnd2, Bcl2, Cflar, Foxf1, Foxl1, Prdm1, Jag2, Grem1, Foxm1, Bmi1, Lgr5, CD44, CD133, Snai1, Snail2, Zeb1, Zeb2, Twist2, Foxc2 [113, 141, 216, 228]. In recent years the application of microarray and High Throughput Sequencing technologies have allowed the rapid and comprehensive understanding of Hh-regulated genes in different tissues and cell lines. In fetal prostate, more Hh-signaling responsive genes, which include Igfbp6, Igfbp3, Fbn2, Ntrk3, Agpt4, Dmp1, Hsd11b1 and Mmp13, have been characterized. Hh-regulated gene sets have also been established in myocardium, gastric cancer cells, hippocampal neurons, and the primary dentition [24, 97, 181, 270, 273]. We retrieved a series of published Hh-signaling downstream gene data sets, including Hh-ligand responsive, as well as Gli1 and Gli2 responsive genes from high throughput sequencing data set as reference (Table III-1).

Biological Functions

Some functions of Hh-signaling were found long before the molecular components of the pathway were discovered. The famous Cyclops-like birth deformation, foetal hypophysial aplasia, foetal thyroidal and adrenal hypoplasia, and foetal gonadal hypertrophy accompanied with midline differentiation abnormality of internal organ and hindbrain, of sheep offspring from ewes that graze on corn lily (*Veratrum californicum*) during 11 to 14 days of pregnancy was found to be due to the alkaloid cyclopamine enriched in *Veratrum californicum* [242]. Cyclopamine was later found to be an Hh-signaling inhibitor capable of binding to and inhibits SMO [36]. Interestingly, the sheep that consumed *Veratrum californicum* in different stages during their pregnancy also gave birth to offspring with additional different deformation phenotypes involved in organ development retardation and short limbs, indicating Hh-signaling has core functions in embryogenesis and development [242].

Early Drosophila development biology research conducted by Christiane Nüsslein-Volhard and Eric Wieschaus showed that the Hh gene is one of several genes that are important in creating the anterior and posterior parts of Drosophila body segments [8, 17]. Genetic knock out of Hh-signaling components in mice also showed that Hh-signaling is an indispensible signal for bilateral symmetry pattern formatting embryo, organ development, nerve system development, limb development and digit identification [3, 15, 20, 86, 184, 186, 213].

As mentioned before, the Yamanaka factors, consisting of Oct4, Sox2, Klf4, c-Myc, can reprogram somatic fibroblast cells to iPSCs and they are known to be core-reprogramming factors [233]. Bim, an Hh target gene product, was shown to be a replacement for Sox2, Klf4 and c-Myc in reprogramming, and activated Hh-signaling by either Hh ligand or SMO agonist oxysterol, can replace Bim1 expression and generate the ability for cell reprogramming in the presence of Oct4 [159]. The success of reprogramming with only Hh ligand and Oct4 indicates Hh-signaling plays a very important role in cell differentiation lineage control and stemness maintenance.

Similar to other embryonic development pathways, the loss of regulation of Hh-signaling is strongly linked to multiple types of cancer. The Hh-signaling pathway has been shown to be involved in medulloblastoma, bladder cancer, breast cancer, cervical cancer, liver cancer, colon cancer, prostate cancer, gastric cancer, pancreas cancer, head and neck cancer, lymphoma and non-small cell lung cancer [32, 56, 68, 94, 105, 114, 155, 196, 225, 261, 280, 288]. The loss of control of Hh-signaling can take place in several steps, including loss of function mutations in Ptch, gain of function mutations in SMO, and irregular activation of the Gli transcription factor family from other signaling transduction pathways that interact with Gli transcription factors [18, 50, 66, 96, 156, 254, 265]. Ptch and SMO mutations were found in human Basal Cell Carcinoma (BCC), thyroid neoplasm, keratocystic odontogenic tumors and Medulloblastoma [61, 224, 266, 289]. EGFR (Epidermal Growth Factor Receptor)-Protein Kinase C (PKC) signaling, Mitogen-activated Protein Kinases

(MAPK) signaling, Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)-Akt signaling, Transform Growth Factor Beta (TGFβ) signaling, and the p53-signaling pathway are all known to interact with the Gli transcription factor [135, 168, 189, 258, 275, 282]. GDC0449 (Vismodegib) targets SMO and serves as a SMO inhibitor. It was approved by the FDA in the spring of 2012 for autocrine Hh-signaling driven advanced and metastatic BCC [6, 185].

Although autocrine Hh-signaling is well represented in cancer cells of BCC and medulloblastoma, paracrine signaling is also found in several types of cancer like sarcoma, prostate cancer and colorectal cancer [23, 44, 102, 234]. Common prostate cancer cell lines do not have active Hh-signaling, reflected by the lack of proliferative responses to Hh ligand, and anti-Hh treatments; the lack of Hh-signaling transcription targets, like gli1 and ptch1 in response to anti-Hh treatment [277]. In contrast, tumor stroma is fully responsive to Hh ligand from the perspective of gene expression and Gli transcription factor activity [261, 272]. Though tumor stroma proliferation does not respond to Hh ligand or anti-Hh treatment, the anti-Hh treatment can lead to baseline inhibition of Hh-signaling target genes. The tumor size of LnCaP tumorgraft treated with the Hh-inhibitor TAK441 decreased in size without showing any Hh-signaling gene alteration, while the tumor stroma showed significant decrease in Hh-signaling marker genes Gli1 and Ptch1 [102]. Tissue recombination of bladder epithelium and stroma, and prostate cancer cell xenografts, as well as co-culture of prostate cancer cells with myofibroblasts, verified the existence of paracrine Hh-signaling between stromal cells and epithelium or cancer cells [102, 205, 207, 287].

In general, stromal cells express Hh-signaling pathway components and are responsive to Hh ligand. The activation of downstream Gli transcription factor activates the transcription, translation and secretion of growth factors that stimulate surrounding tumor cell proliferation. In the bladder epithelium-stroma tissue recombination study, FGF16 secreted from stroma was shown to drive the epithelium proliferation from infection-induced tissue damage [207]. Besides FGF, Transform Growth Factor family protein, BMP4, also plays an important role in paracrine Hh-signaling regulation and tumor metastasis. In co-culture system with LnCaP and MC3T3-E1 cells, BMP4 induces Shh expression, which stimulates the expression of FGF2 and epidermal growth factor (EGF) expression in MC3T3-E1 cells and these growth factors stimulate LnCaP cell proliferation [171].

Although there is solid *in vitro* and *in vivo* data showing the presence of paracrine Hh-signaling between cancer stroma and the tumor itself, paracrine and autocrine Hh-signaling are not mutually exclusive. As above, the general population of prostate cancer cells don't show Hh-signaling responsiveness, while selected cancer stem cells, in prostate cancer, lung cancer, and breast cancer are significantly inhibited in sphere formation, colony formation or self-renewal by Hh-signaling inhibitors [30, 58, 64, 236, 278]. It is not surprising to see Hh-signaling carry the function of cancer stem cell self-renewal considering its core function in iPSC induction.

To summarize, Hh-signaling is an important pathway in multiple aspects of stem cell biology, development biology and cancer biology. The fundamental role of both Esrrb and Hh-signaling in iPSC reprogramming, as well as embryonic development indicates Esrrb and Hh-signaling can have functional overlap.

PART III: PROJECT OBJECTIVES

Esrrb has been shown to be a significant factor in embryonic development, embryonic stem cell self-renewal, induced pluripotent stem cells reprogramming, metabolism, antioxidant responsive gene expression regulation through Keap1-Nrf2 pathway, and estrogen receptor signaling regulation. Esrrb is also important in tumor development. Transcriptionally, Esrrb function has been known from Esrrb knock down experiments followed up with microarray analysis and Chromatin Immunoprecipitation (ChIP) in mouse ESCs and iPSCs, but little is known about Esrrb transcriptional function in cancer cells.

In Chapter II, we establish a model cell line expressing Esrrb and use next generation sequencing (NGS) to perform mRNA profiling to understand the target gens of Esrrb. We distinguished Esrrb ligand dependent and ligand independent target genes for the first time. Using different bioinformatics tools, we classified Esrrb target genes by function enrichment through Gene Ontology (GO); by transcription factor promoter binding motif - gene promoter sequence query; as well as by gene set comparison with known gene sets for data mining.

Hedgehog (Hh) signaling is another core signaling pathway in ESC cell fate regulation and iPSC induction. Its roles in embryogenesis, cancer development, iPSC biology indicate Hh-signaling and Esrrb can interact with each other and have functional overlap. In Chapter III, we establish an Hh-responsive cell line expressing Esrrb and quantify the mRNA expression in its transcriptome by NGS. By categorizing differentially expressed genes (DEGs) based on each DEG behavior in different biological condition, we found genes that are redundantly regulated by either Esrrb or Hh-signaling pathway. Most interestingly, we also found a group of genes that Esrrb and Hh-signaling co-regulate.

In Chapter IV, we tested the effect of Hh-activated SMO, DY131 and Esrrb on Akt phosphorylation and whether DY131 and Esrrb can modulate Akt phosphorylation. We found novel activates of both Esrrb and DY131 to inhibit Akt phosphorylation

Chapter V summarizes our research and infers future research directions.

We also found the anti-Hh-signaling activity of a South African botanical *Lessertia Sutherlandia* from a compound screen and characterized Sutherlandia extract induced global gene expression alteration as well as its regulatory function in

Hh-signaling target genes inhibition. The results and discussion regarding this part of research is summarized in Appendix.

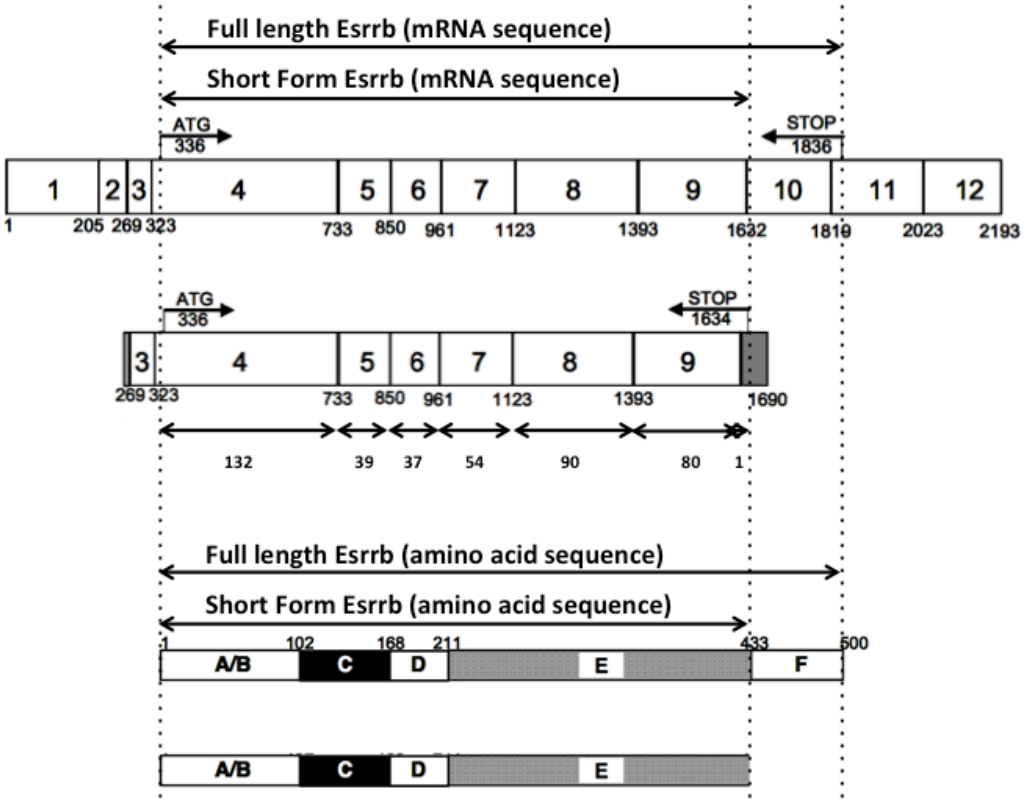


Figure I-1 Molecular structure of full length human Esrrb and Short-form human Esrrb

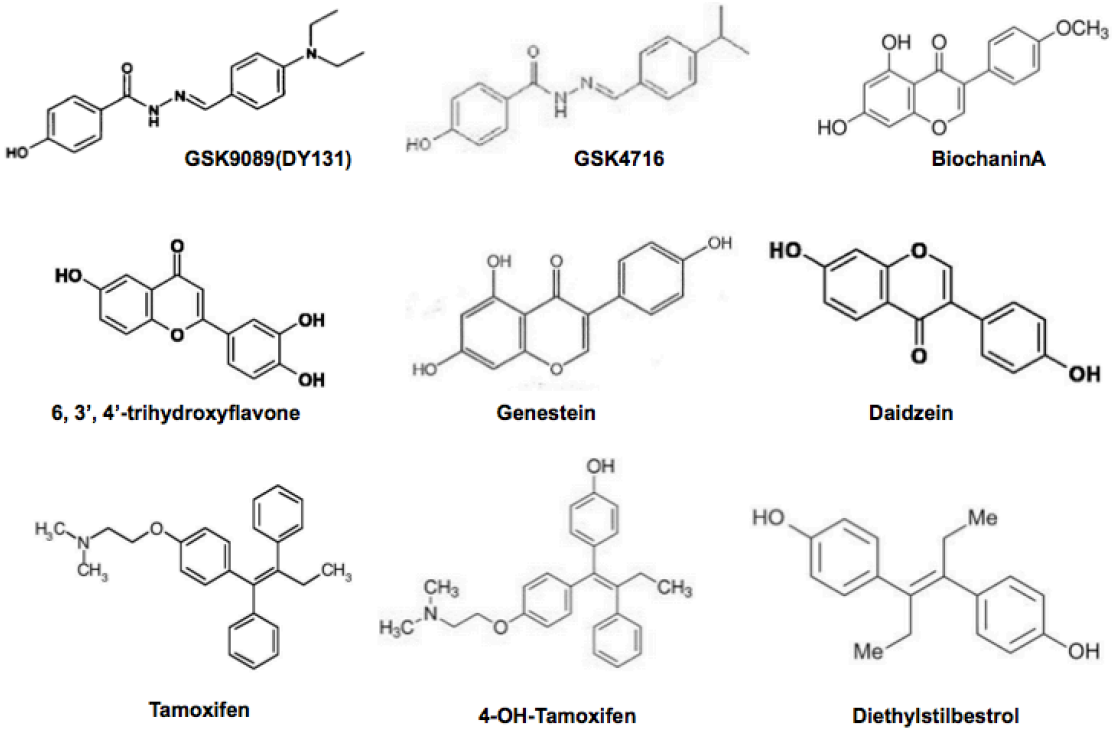


Figure I-2 Structure of Esrrb activity modulators

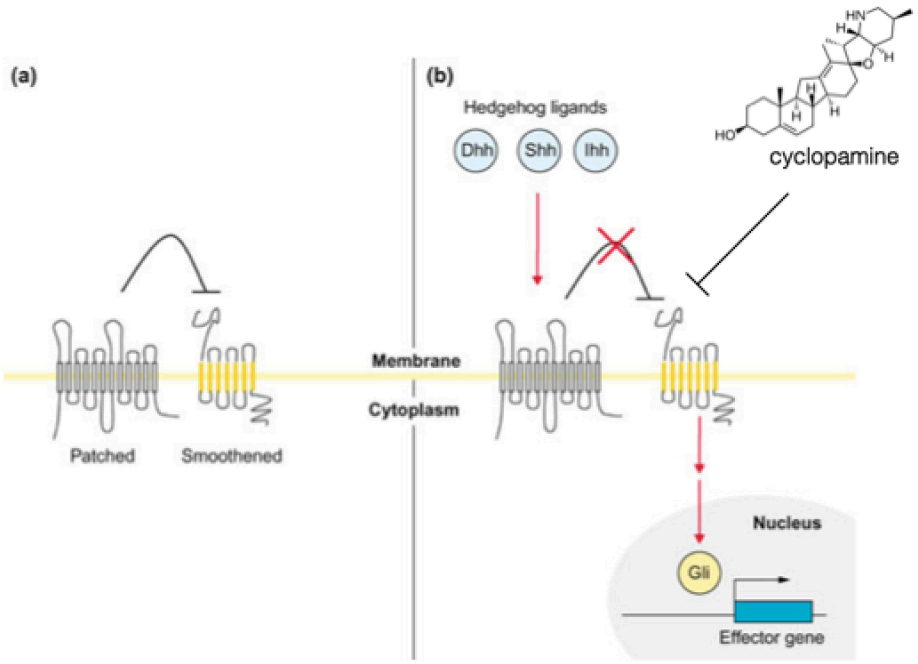


Figure I-3 Illustration of Hedgehog-signaling transduction pathway

When Hh ligand is absent, Ptch inhibits SMO function, and Hh-signaling pathway is not activated. When Hh ligand is present, Hh binds to Ptch and relieve its inhibitory function on SMO. Activated SMO turns on the downstream Hh-signaling and Gli transcription factor translocates into to nuclear and turn on/off Hh-signaling target genes.

Compound	Assay	Activity	Reference
DY131 (DY131)	ERE reporter Gal4 reporter	Agonist	[79, 271, 286]
GSK4716	ERE reporter Gal4reporter	Agonist	[271, 286]
BiochaninA	ERE reporter Yeast two hybrid (ERRβ-PNRC)	Agonist	[219]
6, 3', 4'-trihydroxyflavone	ERE reporter Yeast two hybrid (ERRβ-PNRC)	Agonist	[219]
Genestein	ERE reporter Yeast two hybrid (Esrrb-PNRC)	Agonist	[219]
Daidzein	ERE reporter Yeast two hybrid (Esrrb-PNRC)	Agonist	[219]
Tamoxifen	FRET (Esrrb-SRC1) Yeast two hybrid (Esrrb LBD-SRC1)	Antagonist	[49]
4-OH-Tamoxifen	FRET (Esrrb-SRC1) Yeast two hybrid (Esrrb LBD-SRC1) FRET (Esrrb-RIP140)	Antagonist	[49, 239]
DES	FRET (GRIP1-Esrrb) ERE reporter FRET (SRC1-Esrrb) Yeast two hybrid (Esrrb LBD-SRC1)	Antagonist	[49, 240]

Table I-1. Summary of Known Esrrb ligands

Nucleotide change	Amino Acid change	References
329C→T	A110V *	[46]
872G→T	R291L	[199]
913T→C	Y305H	[9]
959T→C	L320P	[46]
1018_1020delGAG	E340del	[125]
1018_1024dupGAGTTTG	V342GfsX44	[46]
1024G→T	V342L	[46]
1040T→C	L347P	[46]
1156C→T	P386S**	[46, 199]
1166C→T	T389M	[46]
1237G→A	V413I	[199]

*Only mutation found so far in DBD ** Polymorphism

Table I-2 Summary of known hearing loss related mutations found in Esrrb

CHAPTER II

Messenger RNA profile analysis deciphers new Esrrb transcription targets in prostate cancer cells

Abstract

Orphan nuclear receptor Estrogen Related Receptor β (Esrrb or ERRβ), has became a hot research topic in recent years. Although the function of Esrrb is relatively well known in stem cells and early embryonic development, little is known about its function in cancer. Here we investigate the mRNA profile alterations induced by Esrrb expression and its synthetic ligand DY131 in Esrrb null human prostate cancer cells via RNA-seq analysis. We distinguished 67 mRNAs differentially regulated by Esrrb alone, and 1161 mRNAs altered by treatment of DY131 in the presence of Esrrb, indicating Esrrb has both ligand independent and ligand dependent activity. DY131 alone did not alter any mRNA when Esrrb was not expressed, indicating DY131 is a very specific Esrrb ligand. DY131 was found to further regulate 15 Esrrb-altered mRNAs, and acts as an antagonist for 11 of 15 mRNAs and as an agonist for 4 of the 15 mRNAs. From Esrrb regulated mRNAs, we established a hypothetical Esrrb transcription regulation model by matching the DNA motif recognized by transcription factors to the promoter sequences of genes corresponding to altered mRNAs. From our model, we found Esrrb-regulated mRNAs that are potentially mediated by these transcription factors: Tagln, KIAA1199, Ddx60, Nrip3, Creb5 and Hsf4. Gene ontology analysis showed altered components in several cellular processes in regards to transcription and translation regulation, cell proliferation and apoptosis regulation, and cellular metabolism. In addition, we found p53 C-terminal phosphorylation is DY131 activated and Esrrb dependent, further revealing the mechanism of p21 regulation by Esrrb. Our results characterized for the first time mRNA profiles driven in DU145 prostate cancer cells by Esrrb expression and

Esrrb ligand DY13 and provide multiple markers to characterize Esrrb's function in future research.

Introduction:

Esrrb encodes nuclear receptor Estrogen Related Receptor β (Esrrb), which belongs to the nuclear receptor family. Esrrb acts as a transcription factor by binding to a specific DNA sequence Estrogen Related Receptor Response Element (ERRE), also known as Steroid Factor Response Element (SFRE), or half site Estrogen Response Element [82, 246].

Esrrb, first cloned in 1988, was not intensively studied until recent years. In animal studies, the Esrrb knock out is embryonic lethal due to placental malformation [137]. Though early studies showed a very limited range of tissues with positive Esrrb expression, recent studies reported that the short form Esrrb alternative splicing isoform, lacking the last 3 exons, has a broad range of expression [284]. Esrrb was found to be a core-reprogramming factor for inducing Pluripotent Stem Cells (iPSCs) [70, 137, 260, 284]. c-myc and klf4 of the OSKM (oct4, sox2, klf4, c-myc) core-reprogramming factors can be replaced by Esrrb [70, 260]. Esrrb was also recently reported to drive Sox2 transcription and induce iPSC in a single cell system [19].

Tumorigenesis and tumor progression are related to Esrrb. Esrrb was shown to be down-regulated in prostate cancer compared to normal prostate tissue [41, 78, 274]. Its re-expression in DU145 and LNCaP cells was shown to stimulate tumor suppressor CDKN1A (p21) concentration. Also Esrrb can inhibit Estrogen Receptor transcriptional activity in uterine endometrial cancer cells and Nrf2-Keap signaling pathway in breast cancer cells [16, 285].

There are a handful of transcriptome-wide expression survey data from Esrrb knockdown in both human iPS cells and mouse embryonic stem cells [39, 172, 174, 179]. Other than Klf4, c-Myc, Cdkn1a and Cyp19a1, the primary target genes controlled by Esrrb in cancer cells are still not well known. Esrrb binding sites across the genome of iPS cells were reported in 2012, but there is not enough information to link the binding sites to the genes that these binding sites regulate in other model systems like cancer [174]. Thus, we performed RNA-seq analysis to characterize Esrrb regulated mRNAs in a prostate cancer cell line. We propose a model of how Esrrb regulates differentially expressed genes by transcription factor-promoter query. We found the treatment of DY131, an Esrrb synthetic ligand, expands Esrrb's transcriptional regulation activity to many more genes within a prostate cancer cell transcriptome.

Materials and Methods

Cell culture and reagents

DU145 (ATCC Number: HTB-81), TRAMPC2 (ATCC Number: CRL-2731) and HEK293 (ATCC Number: CRL-1571) cells were obtained from the American Type Culture Collection (ATCC). DU145 and TRAMPC2 cells are cultured in RPMI1640 media (Invitrogen, Grand Island, NY) with 10% Fetal Bovine Serum (FBS) (GE Healthcare Life Sciences, Logan, Utah). HEK293 cells are cultured in Eagle's Minimal Essential Medium (DMEM) (Invitrogen, Grand Island, NY) with 10% FBS. 70% confluent DU145 or TRAMPC2 cells are transfected with either pcDNA3.1-zeo (+)-Esrrb expression vector [284], or control empty vector pcDNA3.1-zeo (+) (Promega, Madison, WI). Empty vector or Esrrb expression vector transfected DU145 cells are maintained in medium containing 150ug/ml Zeocine (Invitrogen, Grand Island, NY) for 3 weeks for selection. The stably transfected DU145 cells carrying Esrrb trans-gene are named DU145-Esrrb, while the control DU145 cells transfected with the empty vector are called DU145-pc3.1. Total RNA and protein are collected from cells after they are confluent in 60mm petri dishes, cultured with phenol-red free RPMI1640 with 10% Charcoal-stripped FBS [259]. For DY131 (Tocris Bioscience, Bristol, UK) treatment, cells are plated in 60mm petri dishes until confluent; DU145-pc3.1 and DU145-Esrrb are incubated with 3uM DY131 diluted in medium with charcoal-stripped FBS for indicated length of time.

Western-blot

DU145-pc3.1 and DU145-Esrrb were incubated with 3uM DY131 diluted in medium with charcoal-stripped FBS for indicated length of time. TRAMPC2 cells were

culture with indicated concentration of DY131 for 24 hours. DU145-Esrrb cells were transfected with pGFP-V-RS based scrambled shRNA or 3 different shRNAs against Esrrb (Scrambled shRNA control: GCACTACCAGAGCTAACTCAGATAGTACT; Esrrb shRNA #1: CAACTCAGACCATTCCAC GGAGGCATCCT; Esrrb shRNA #2: GGCTGCTGAACAGGATGTCCTCGGACGAC; Esrrb shRNA #3: CCAAGATTGTCTCATACCTACTGGTGGCT) (Origene, Rockville, MD) with Fugene HD (Promega, Madison, WI) for 18 hours, followed with 24 hours treatment of 3uM DY131. Total protein was isolated from DU145-pc3.1, DU145-Esrrb and TRAMPC2 cells. Total protein was isolated and 20ug protein was loaded on 9% SDS gels. After the proteins were transferred to nitrocellulose membrane, the membrane was blocked and then incubated with 1:1000 diluted polyclonal anti-p53Ser392 rabbit IgG (Cell signaling, Beverly, Massachusetts, Cat.No: 9281), 1:2000 diluted monoclonal anti-Esrrb mouse IgG (R&D system, Cat. No: PP-H6705-00), 1:1000 diluted polyclonal anti-p21 rabbit IgG (Santa Cruz, Dallas, TX, Cat. No: H164), 1:2000 diluted monoclonal p53 (DO-1) mouse IgG (Santa Cruz, Dallas, TX, Cat. No: sc-126) and 1:2000 diluted polyclonal anti-GPADH rabbit IgG (Santa Cruz, Dallas, TX, Cat. No: sc-25777) at 4 degrees overnight. The membranes were then washed and incubated with anti-mouse or anti-rabbit secondary antibody, and chemoluminescence (Promega, Madison, WI) signals were collected by using x-ray films (Fisher Scientific, Pittsburg, PA).

Reverse Transcriptase PCR and quantitative PCR

Total RNA was isolated and purified from DU145-pc3.1 and DU145-Esrrb using RNeasy kit (Qiagen, Venlo, Netherlands). 1000ng of total RNA was used to create cDNA libraries using Superscript III Reverse Transciptase with random primers and oligodT (Invitrogen, Grand Island, NY). Esrrb mRNA concentration was determined using quantitative PCR (qPCR) (iQ SYBR, BioRad, Herculus, CA) on ABI7500 system (Applied Biosytems, Foster City, CA). PCR reaction condition: 95 degree, 30 seconds; 60 degree, 40 seconds; 72 degree, 40 seconds. Primer sequences: ZCWPW2 (Genbank: NM_001040432): Forward primer: AACAGGGTTGTCTGTGAGACGGA; Reverse primer: TGCAGGAGCTTCTGGGCTGC. HOXB8 (Genbank: NM_024016): Forward primer: GATGCGCC CGCAAGCAGC; Reverse primer: CCCAGGGCGTGCGATACCTC. TAGLN (Genbank: NM_001001522): Forward primer: ATGCCCCGGATGACTTGGCT; Reverse primer: GCCATGTCTGGGGAAAGCTCCT. F13A1 (Genbank: NM_000129): Forward primer: TGTTCCGTGAAATCCGGCCC; Reverse primer: TGCACGTCCAG CTCGCCATA. PXDN (Genbank: NM_012293): Forward primer: GCAAGCATTTAA GGGACTTGCCTCT; Reverse primer: GCAAAAATAGCCTCTCGAGCTTCGG. AOX1 (Genbank: NM_001159): Forward primer: TACGTGAACGGCCGCAAGGT; Reverse primer: TGGCTGGGTGATGCCTTATCCT. BMP4 (Genbank: NM_001202): Forward primer: CCACCACGAAGAACA TCTGGAG; Reverse primer: GCCCCTTTCCCAATCAGGGC. TGFβ: (Genbank: NM_000660) Forward primer: AGTGGACATC AACGGGTTCAC; Reverse primer: CGCACGCAGCAGTTCTTCTC GAPDH: (Genbank: NM_001256799); Forward primer: ACCCACTCCTCCACCTTTG; Reverse primer:

CTCTTGTGCTCTTGCTGGG. Esrrb: (Genbank: NM_004452) Forward primer: CAAGAAGCTCAAGGTGGAGAAGGAGGAG; Reverse primer: CGGTCTGTCC GTTTGTCTGTCTGTAGGT. Esrrg: (Genbank: NM_001134285) Forward primer: ACCATGAATGGCCATCAGA A; Reverse primer: ACCAGCTGAGGGTTCAGGTAT.

Deep sequencing and Differentially Expressed Genes

2500ng total RNA from 2 biological replicates was used to generate cDNA libraries using TruSeq Stranded mRNA Sample Preparation kits (Illumina, San Diego, CA) according to the manufacturer's manual. RNA quality and fragment sizing of cDNA library were determined by the University of Missouri DNA core. Deep sequencing was performed by the MU DNA core using Illumina HiSeq 2000 following the manufacture's instruction. Briefly, all samples (8 total) are pooled into one lane with each sample was annealed to a specific indexed adaptor. 50bp single-end-reads were generated. For each sample, around 18 million reads were generated in .fastq format. The sequencing reads were trimmed and filtered using FASTX-Toolkit (V 0.0.13) (http://hannonlab.cshl.edu/fastx_toolkit), and mapped to genome (USCS hg18) using Bowtie and TopHat [122, 237]. Gene expression values are determined by gene raw read counts using an in-house tool MULTICOM-MAP[221-223]. Raw reads were normalized to each sample's library size and differentially expressed genes were calculated by edgeR [194]. Specifically, we kept the genes that have at least 1 count-per-million (cpm) in at least 2 groups and compute effective library sizes.

Pairwise gene expression tests were carried out by exact test and differentially expressed genes were determined by log2 Fold change (Log2FC) (Log2FC>1, or Log2FC<-1), p value ($p<0.05$) and False Discovery Rate (FDR<0.05) [128].

Gene set Function Enrichment

Gene Ontology (GO) analysis was performed using DAVID bioinformatics sources 6.7 [98, 99]. Differentially expressed genes from certain pairwise comparisons were uploaded to DAVID server (http://david.abcc.ncifcrf.gov) and GO analysis were performed for Biological Process (BP). Minimum counts were set as default value (2 counts) and maximum EASE score (p-value) was set to 0.05. Differentially expressed genes Pathway enrichment analysis was performed by Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway [109, 110]. Spearman Ranking Correlation is analyzed and made by R (version 3.0.2). Gene expression heat map and hierarchical clustering were created by R/Bioconductor (version 2.13) package gplot.

Esrrb gene regulation network

The altered mRNAs are used as candidate genes for the regulation network construction. We retrieved the transcription factors from the Animal Transcription Factor Database (Animal TFDB) (http://www.bioguo.org/AnimalTFDB/index.php). For every transcription factor, R/Bioconductor package MotfiDb(1.4.0) and BiomaRt(2.18.0) were used to retrieve the binding motif of transcription factor and gene

upstream flanking region sequence respectively [62, 63]. If a transcription factor does not have a known DNA binding motif, it is discarded for further analysis. The network was constructed by querying transcription factor binding motifs against all retrieved promoter sequences (Figure II-7). If a transcription factor (source node) binding motif matched with the promoter sequence of another transcription factor (terminal node), a directed edge (arrow) is drawn to point to terminal node, indicating the terminal node gene has potential binding sequence(s) of source node. An in-house Perl script (available upon request) was generated to construct the relationships among the transcription factors and visualization of the regulatory network is processed through Cytoscape (http://www.cytoscape.org).

Statistical analysis

Unless indicated otherwise, all qPCR experiments were performed in triplicate and reported differences were statistically significant ($p < 0.05$; t test). Statistical significance is represented by $p<0.05$. Statistical significance of gene set overlap (Venn Diagram) is tested according to previous reported method [283].

Results

Establishment of the Esrrb stably transfected DU145 cells.

We transfected Esrrb expression vector or control pcDNA3.1 (Zeo+) vector into DU145 cells. After 3 weeks of Zeocine selection, we characterized the Esrrb status by

Reverse Transcriptase (RT)-PCR, qPCR or western blot analysis (Figure II-1 a, b, c). Our results showed that Esrrb was successfully expressed in DU145-Esrrb cells. Although DU145 has been reported to express basal concentration of Esrrb by other group, the Esrrb concentration is below the detection limit of RT-PCR and western blot; however, RNA-seq showed that DU135-pc3.1 cells have a small amount of Esrrb expressed. Compared to HEK293 cells, which express endogenous Esrrb, its overexpression in DU145 cells raised the Esrrb protein concentration to a comparable physiological concentration (Figure II-1b). In addition, our RT-PCR results and RNA-seq results confirmed the Estrogen Related Receptor Gamma (Esrrg) null status of DU145 cells, and eliminated any possible functional contamination by Esrrg in our Esrrb studies (Figure II-1c).

Esrrb-driven mRNA profile alteration and Esrrb regulated gene network.

To distinguish genes regulated by Esrrb at the mRNA level, we performed RNA-seq analysis on cDNA libraries constructed from 2 biological replicates of both stable DU145-pc3.1 and DU145-Esrrb cells. Transcriptome comparison using Spearman Ranking Correlation analysis showed that the expression of Esrrb in DU145 created a distinct transcriptome compared to control DU145 cells (Figure II-2a). We found 67 genes altered (more than 2 fold change, FDR less than 0.05). Among the altered mRNAs, 21 genes were up-regulated, 46 genes were down-regulated (Figure II-2b, Table 1). 7 genes that are among the most changed genes (ZCWPW2, HOXB8, TAGLN, F13A1, PXDN, AOX1, and BMP4, as well as TGFβ as a negative control)

were confirmed by qPCR (Figure II-3). Gene Ontology (GO) analysis shows that the products of Esrrb driven differentially expressed genes fell into functional categories of regulation of cell development as well as immune responses (Table II-2), although pathway annotation by KEGG did not return significant hits on known pathways (Data not shown).

We searched ERRE sequence in the upstream sequence of these 67 genes. We set the region to be searched to 2000 base pairs upstream of the coding sequence. Among these candidate genes, there are 20 genes that carry at least one copy of ERRE in their 2000 nucleotide upstream sequence of coding sequence. Interestingly, there are 3 genes (COX6B2, TAGLN, NEBL) that have an ERRE in their proximal promoter region (-250 base pairs). 4 genes carrying the Esrrb binding motif are transcription factors KIAA1199, NRIP3, TAGLN and DDX60, which serve in our model as mediators for Esrrb-driven mRNA alteration (Figure II-5a). The presence of transcription factors among altered mRNAs indicates that Esrrb is a master transcription regulator that transduces its function to more genes by inducing the expression of other transcription factors.

DY131 regulates Esrrb to affect mRNA concentration.

To get a more comprehensive understanding of Esrrb-regulated mRNAs and characterize Esrrb's potential ligand dependent activity, control DU145-pc3.1 and DU145-Esrrb cells are treated with the Esrrb/Esrrg synthetic ligand DY131. Since both

qPCR and RNA-seq show Esrrb transcript concentration is extremely low in DU145 cells and Esrrg is absent, and Esrrb protein concentration is also below the detection limit of western-blot analysis, it is not surprising to see DY131 treatment did not result in any qualified differential expressed gene, indicating DY131 is a very specific ligand for Esrrb (Figure II-4a). After we applied DY131 to DU145-Esrrb cells, we found the DY131 treatment with Esrrb expression greatly modifies the transcriptome, making it quite different from that of DU145-pc3.1 or DU145-Esrrb cells (Figure II-2a). We detected 1161 altered mRNAs by applying DY131 to DU145-Esrrb cells (861 down-regulated, 300 up-regulated), and 15 of them overlapped with Esrrb-induced mRNA alterations (Figure II-4b, c, Table II-3). By comparing the trend of the mRNA changes induced by Esrrb expression and DY131 treatment, DY131 acts as an agonist for 4 of the genes, and an antagonist for 11 of the genes (Figure II-4d). Interestingly, there are another 1146 mRNAs changed compared to Esrrb alone, indicating the ligand-dependency of their responses (Table II-3).

GO analysis showed the up-regulated mRNAs important for regulation of transcription, regulation of apoptosis and proliferation, while a majority of down-regulated genes are related to oxidation and reduction, metabolism and translation elongation (Table II-4, Table II-5). Based on the novel network concept we introduced, we proposed another Esrrb gene expression regulation network in prostate cancer cells to reflect the broader effect DY131-activated Esrrb has on transcription factor expression (Figure II-5b). We found that the DY131-activated Esrrb-regulated gene network is mediated by the four transcription factors including Hsf4, Tagln, Creb5 and

Egr1, characterized by the number of input edge and their direct relationship to Esrrb, as well as number of output edges.

Discussion:

Esrrb has gained lots of attention in recent years because of its biological function in stem cells and its ability to reprogram somatic cells to iPSC with Oct4 and Sox2 [26, 39, 70, 218, 241, 279, 283]. Several other functions of Esrrb have also been discovered including altering energy balance, estrogen receptor and glucocorticoid receptor transcription function modulation, keap1-nrf2 signaling inhibition, and tumorigenesis in prostate cancer and endometrial adenocarcinoma [16, 21, 22, 78, 80, 253, 274, 285]. But genome-wide and transcriptome-wide Esrrb function and Esrrb-regulated genes in cancer cells are not well studied.

Esrrb has been reported to be constitutively active in the absence of a ligand and this is supported by the evidence that Esrrg, which shares over 80% of its Ligand Binding Domain with Esrrb, has a transcriptional active conformation compared to E2 activated Estrogen Receptor by x-ray crystallography [1, 87, 88, 164]. Another explanation for this endogenous activity is that Esrrb binds to an unknown endogenous ligand and regulates gene expression.

The endogenous ligand hypothesis is supported by a report that culturing the cells with charcoal-stripped serum-containing medium can eliminate the transcriptional activity of Esrrb on SFRE/ERRE [220, 245]. In our assay, transfected cells were plated 48 hours in medium supplemented with charcoal-stripped serum before RNA extraction. This should have removed Esrrb endogenous ligand and our data with DY131 ligand clearly showed that Esrrb has both ligand-independent and ligand-dependent activity.

Esrrb has been reported by Chan et.al to be a tumor suppressor in DU145 and LNCaP prostate cancer cells using both *in vitro* and *in vivo* models [274]. Expression of Esrrb induced p21/WAF1 by directly binding to an ERRE in p21's promoter and caused cell cycle arrest at S-phase and significantly inhibited the cell growth [28, 274]. Interestingly, we did not find p21/WAF1 significantly changed after Esrrb expression alone, but after we treated DU145 cells with 3uM DY131, we observed a significant increase of p21/WAF1 mRNA (Table II-3, Figure II-8). On the other hand, knocking down the overexpressed Esrrb in DU145-Esrrb cells significantly decreased p21 protein concentration increase in response to DY131 treatment (Figure II-6b). Scrutinizing the data revealed that Chan's lab cultured their cells with full serum, while we used charcoal-stripped serum for cell culture and DY131 treatment before RNA isolation [274]. This supports the hypothesis that Esrrb has an endogenous ligand, which is potentially a hydrophobic molecule that can be removed by charcoal treatment.

p21 is a p53 transcriptional target. Although published data shows that Esrrb regulates p21 expression by directly binding to the promoter of p21 independent of p53 activity, the presence of a group of p53 target genes after DY131 treatment to Esrrb expressing cells, suggesting ligand bound Esrrb may regulate p21 in a manner involving p53 (Figure II-8) [111]. Although p53 mRNA and protein concentrations do not respond to either Esrrb alone or DY131, the combination of both induced a rapid increase of p53 Ser392 phosphorylation (Figure II-6a). In another Esrrb null prostate cancer cell line, TRAMPC2, transient transfection of Esrrb generates similar inductive effects on p53 phosphorylation. In empty vector transfected TRAMPC2 cells, DY131 has little effect on p53 phosphorylation. However, when Esrrb is expressed, DY131 dose dependently increased p53 C-terminal phosphorylation. Similar to the p21 response, knocking down Esrrb with shRNA against Esrrb in DU145-Esrrb cells resulted in a loss of response of p53 phosphorylation to DY131 treatment. Thus Esrrb could potentially modulate p53 transcriptional regulation activity and indirectly p53's downstream target genes, including p21 (Figure II-6, Figure II-8).

From the Esrrb-regulated gene list, we found a few target genes that are related to the known function of Esrrb. KIAA1199 encoded gene product has been shown to associate with cellular mortality, and more interestingly, a KIAA1199 mutation is reported to relate to nonsyndromic hearing loss. Considering the significant effect of Esrrb mutations on human hearing loss, KIAA1199 could be a mediator of Esrrb mutant related hearing loss [2, 89, 92, 151], supporting the report that Esrrb mutations are related to human recessive hearing loss by linkage analysis [9, 46, 125, 199]. Another

interesting Esrrb responsive gene is Tagln (Transgelin), which is inhibited by Esrrb and DY131 treatment relieves the inhibition. Tagln is reported to promote DU145 cell migration and invasion, indicating Esrrb can also affect DU145 cell behavior by acting through Tagln [123].

Judging by the numbers of altered mRNAs induced by Esrrb with or without DY131, and that DY131 did not alter any mRNA in the absence of Esrrb, we conclude that DY131 is very specific for Esrrb, when Esrrg is not present. Besides Esrrb and Esrrg, DY131 has also been reported to bind to the Hedgehog (Hh) signaling pathway component Smoothened (SMO), and mediate active SMO ciliary translocation and SMO inhibition [255]. DU145 cells have SMO expression (Figure II-9), but we did not find any Hh-signaling regulated mRNA changes in Gli1, Ptch1 or Hsd11b1 in the mRNA profiling, thus indicating the absence of an active Hh-signaling pathway. Alternatively, SMO may not be in the conformation or subcellular location required for DY131 binding.

To comprehensively understand the biological meaning of the transcriptome changes with Esrrb and DY131, we performed GO analysis to enrich the function of Esrrb and DY131 represented by known functions of altered mRNAs. As a transcription factor, DY131-activated Esrrb altered the expression of a significant number of other transcription factors like homeobox B5, B6 (HOXB5, HOXB6), zinc finger proteins (ZFPs) and SOX9; and chromatin remodeling factors like Histone deacetylase 5

(HDAC5), Nucleophosmin (NPM1) and Integrin beta 3 binding protein (ITGB3BP), revealing a role as a master regulator of transcription (Table II-3).

To fully present the interrelationship among the differentially expressed genes in our data set and discover direct Esrrb downstream genes, as well as help direct future studies, we established a hypothetical gene regulation network by direct transcription factor binding motif screening in differentially expressed gene promoters. Over the past few years, sophisticated tools have been generated to reconstruct gene regulatory networks from gene expression profiles as large amounts of gene expression profile data sets became available [243]. Most of those tools begin with clustering genes based on the degree of the response to stimuli. Although this is reasonable considering the signal amplification along the signaling transduction pathways, the same transcription factor can stimulate the transcription of one set of genes, while also repressing another set of genes at the same time in response to the same cellular stimulus. Viewing mRNA profile as a projection of the function of a higher level of biomolecules interactions, we want to use the genomic feature of altered transcription factors to build a model that: 1) infers the driven mediator for the gene expression profile alteration and 2) directs further molecular function characterization.

Our gene regulation model (Figure II-7) is built based on the theory that transcription factors recognize and bind to their DNA binding motif in the regulatory sequence of their target genes. A caveat is that it does not consider remote transcription

control from enhancer binding and indirect transcription cofactor binding that requires a bridging factor to recognize regulatory sequence. For the data visualization, the edges represent the potential of the start node to bind to the promoter of the target node. After DY131 treatment, transcription regulatory activity of Esrrb expanded to a much larger scale (67 genes versus 1161 altered genes). Using our transcription factor network screening analysis method, this transcriptional regulation expansion is predicted to be controlled mainly by Hsf4, Tagln, Creb5 and Egr1 by counting their relationship with Esrrb and numbers of output edges (Figure II-6). Our innovative gene set enrichment tool is capable of providing information of potential transcription factor binding sites as well as the binding site associated factors. Chromatin-Immunoprecipitation (ChIP), ChIP-seq or Gel Shift assay can be used to validate the results from our *in silico* enrichment. On the other way, out tool can help to determine the factor of interesting to be tested.

We found a few genes that can serve as biomarkers to reflect Esrrb activity. AOX1, PXDN, F13A1, BMP4 and NPTX1 are the top 5 genes that Esrrb inhibits, while ZCWPW2, FGB, TKTL1, PRSS8, ZC4H2 are the top 5 genes stimulated by Esrrb (Figure II-2b, Table II-1). Within the set of Esrrb-regulated genes, a subset of 15 genes can be further regulated by DY131, with DY131 serving as an agonist for 4 genes, and an antagonist for 11 of the shared genes (Figure II-4d, Figure II-10). These genes are thus potential markers for the measurement of Esrrb transcription activity in future studies.

In conclusion, we characterized the transcriptome alteration induced by Esrrb expression as well as Esrrb with its ligand DY131 in prostate cancer cells for the first time and conclude Esrrb has both ligand-independent and ligand-dependent transcriptional activity. We established a model of gene regulatory network construction to predict direct transcriptional targets of a transcription factor that can be tested by experiment. Finally, analysis of Esrrb target genes combined with functional testing of p53 phosphorylation confirms Esrrb regulation of p21 and indicates Esrrb may be an important factor in cancer prevention and treatment.

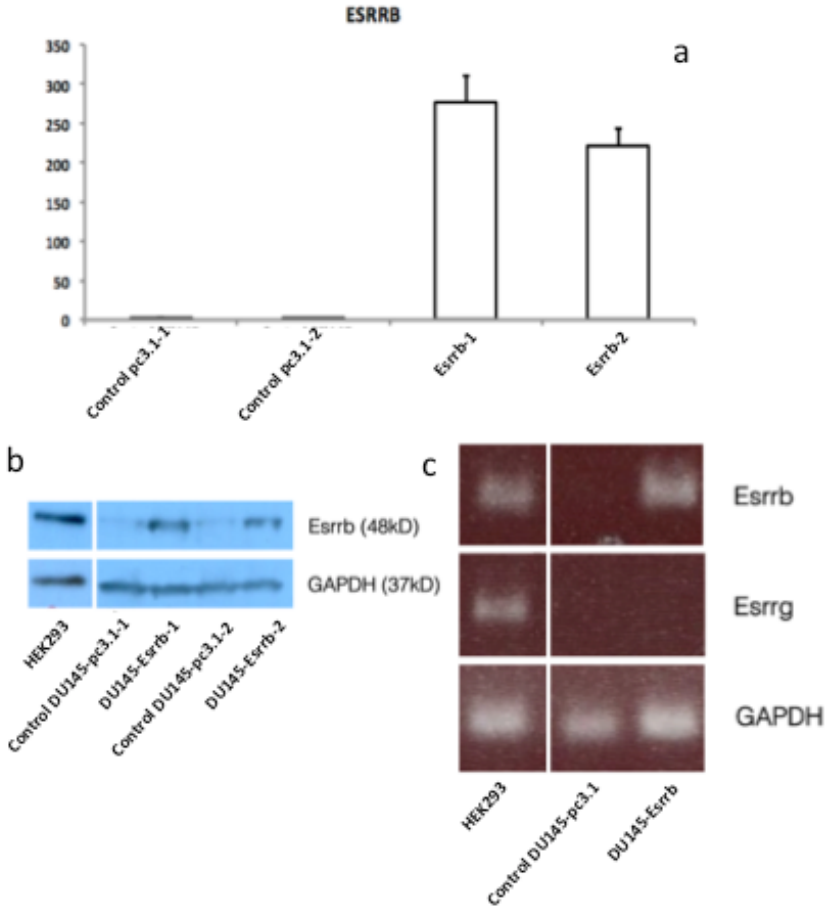


Figure II-1. Characterization of Esrrb-expressing cancer cell line.

Esrrb status of two independent sets of stable transfected control DU145-pc3.1 and DU145-Esrrb cells are tested by (a) quantitative PCR, (b) Western blot and (c) reverse transcriptase PCR. (a) RNA was extracted from two sets of independent stable transfected DU145-esrrb cells and two sets of control DU145-pc3.1 cells, relative mRNA concentrations of Esrrb are measured by qPCR, Esrrb transcripts concentration were determined by standard curve method and Esrrb concentration were first normalized to the concentration of house keeping gene GAPDH, then normalized to Esrrb/GAPDH ratio of DU145-pc3.1 cells. (b) Total proteins are extracted form HEK293, DU145-Esrrb and control DU145-pc3.1 cells. Protein concentration of Esrrb was determined by western blot using GAPDH as internal control. (c) RT-PCR was performed on total RNA extracted from HEK293, DU145-esrrb and control DU145-pc3.1 cells. Esrrb is expressed in DU145-Esrrb cells, while Esrrg is not expressed in DU145 cells.

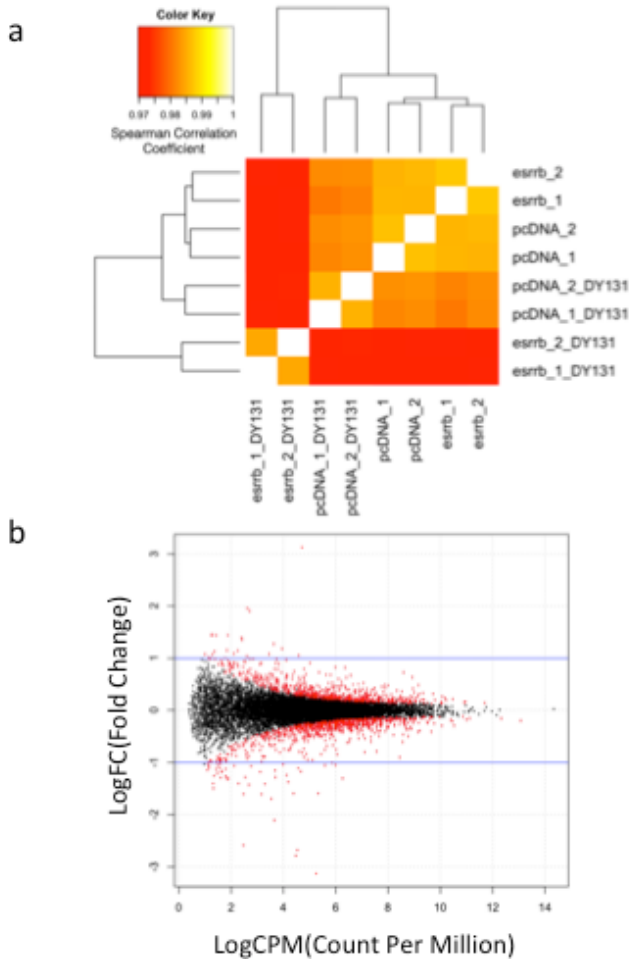
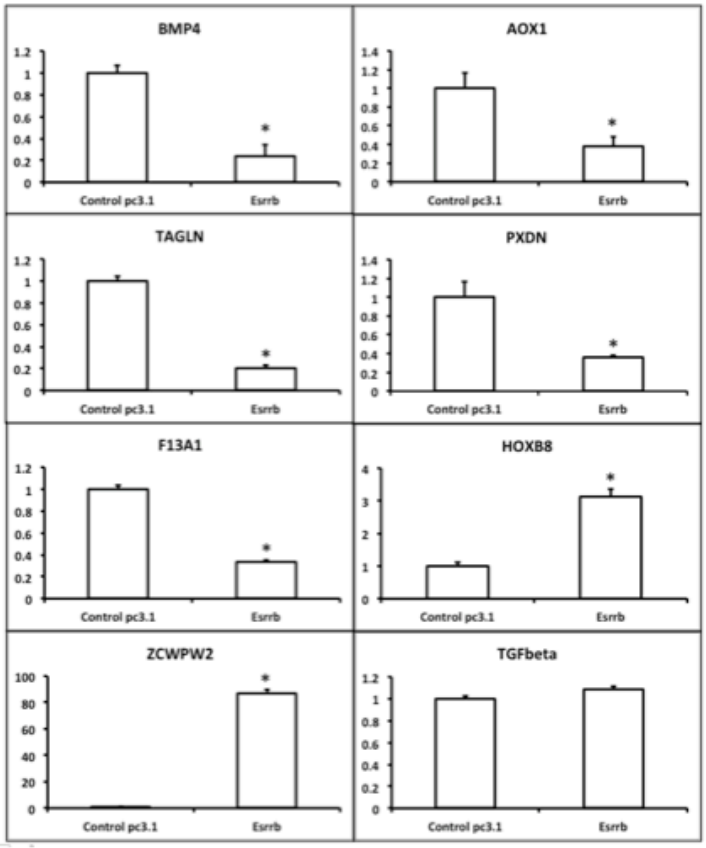


Figure II-2. Transcriptome correlation and Esrrb altered mRNAs.

(a) Transcriptome correlation analysis was performed using Spearman Ranking Correlation. Color represents the correlation coefficient. DY131 treatment to DU145-Esrrb cells results in the lowest correlation coefficient. (b) Plot of Esrrb expression induced gene expression alteration. Genes expressed at adequate level are tested for differential gene expression test. The plot is made by plotting the Log2FC (Fold Change) against the Log2cpm (count-per-million) difference in two different conditions. Red color marks the genes that are significant differentially expressed (FDR<0.05), and the blue lines marked the Log2FC cutoff value (Log2FC>1 or Log2FC<-1). 67 genes passed both thresholds.



Gene Symbol	Fold Change	P-Value
BMP4	0.17	1.7E-29
TAGLN	0.44	5.5E-30
F13A1	0.16	9.3E-114
AOX1	0.09	4.9E-184
PXDN	0.14	4.5E-87
HOXB8	1.97	3.6E-11
ZCWPW2	37.15	3.5E-215
TGFB	N.S.	p>0.05

Figure II-3. Esrrb-regulated mRNA validation.

(Left panel) qPCR validation of Esrrb-regulated mRNAs. Expression values are normalized to Gapdh and normalized ratios are further normalized to that of DU145-pc3.1. Error bars represent standard deviation. Student t test was performed for statistical analysis ($p<0.05$). 7 genes are differentially expressed in both RNA-seq and qPCR, 1 gene, TGFbeta, is not differentially expressed in either assay and serves as negative control. (Right panel) RNA-seq analysis result, Fold Change (FC) indicates the ratio of normalized read counts from Esrrb expressed condition to that of control condition.

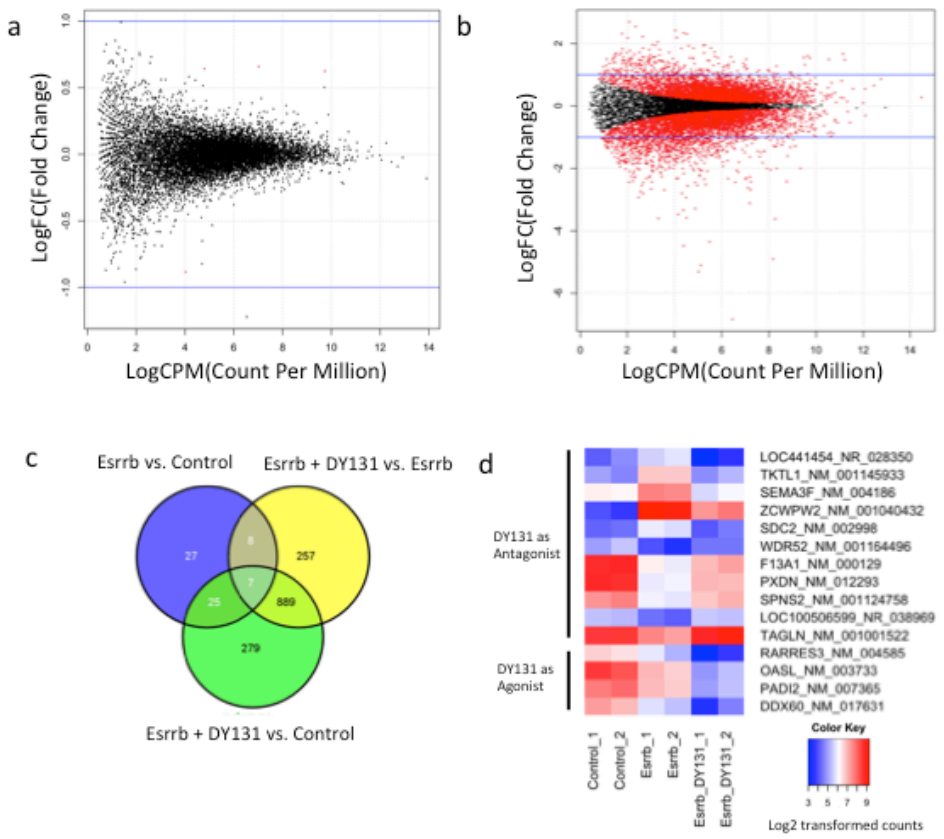


Figure II-4. mRNA alteration by DY131 requires Esrrb expression.

Plot of (a) DY131-induced mRNA alteration and (b) DY131-induced mRNA alteration with Esrrb expression. DY131 altered 4 mNRAs (FDR<0.05), but none of the genes changed more than 2 fold. In contrast, when Esrrb is expressed, DY131 altered 1161 mRNAs. (c) Venn Diagram of pairwise comparisons of altered mRNAs showed 15 (p= 0.0014) Esrrb altered mRNAs can be further regulated by DY131 treatment (overlap between Esrrb vs. control and Esrrb+DY131 vs. Esrrb). (d) Heat map of mRNA concentration of the 15 genes that response to both Esrrb expression as well as DY131 treatment. mRNA corresponding reads counts are normalized and log2-transformed. DY131 is an agonist for 4 mRNAs that are responsive to Esrrb, while it is an antagonist of Esrrb in regulating the other 11 mRNAs.

Figure II-5. Gene regulatory network model initiated by Esrrb.

Gene regulatory network is constructed on differentially expressed transcription factor related genes. The edge (arrow) indicates the source node transcription factor (circle with transcription factor gene symbol, with edges pointing away) has at least one binding motif in the promoter region of the terminal node (circle with transcription factor gene symbol, with edges pointing toward). When DY131 is not added to the cells, a small-scale network is built as (a). However, when DY131 is treated to the Esrrb-expressing cells, the range of gene expression alteration is expanded and a larger network is formed in (b). Creb5, Hsf4, and Tagln are predicted to be core mediators of DY131 bound Esrrb generated network.

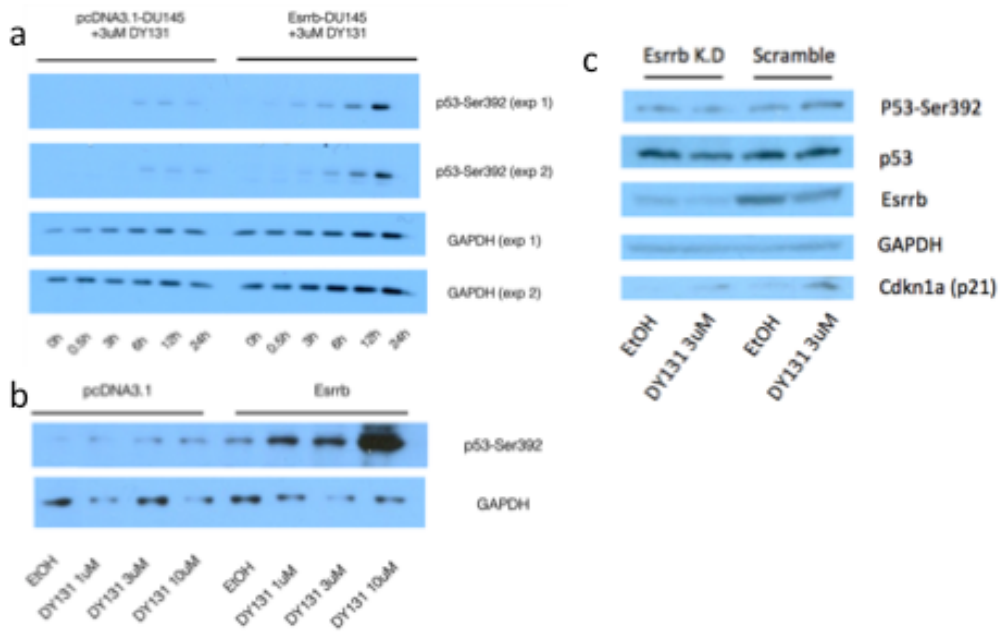


Figure II-6. DY131-activated Esrrb regulates p53-driven gene expression by altering p53 activity.

(a) Time course of p53 phosphorylation at Ser392 in the presence of 3uM DY131.Control pcDNA3.1-DU145 and esrrb-DU145 cells are treated with 3uM DY131 for different time, p53 phosphorylation and GAPDH are measured by western blot. DY131 requires the expression of Esrrb to stimulate p53 phosphorylation. (b) Western blot analysis of p53 phosphorylation and GAPDH in TRAMPC2 cells transfected with Esrrb and treated with different concentration of DY131. (c) Esrrb was knocked down by shRNA against Esrrb. After the Esrrb knock down, DY131 was treated for 24 hours. Both p21 concentration and p53 phosphorylation were decreased compare to scramble RNA transfected counterpart.

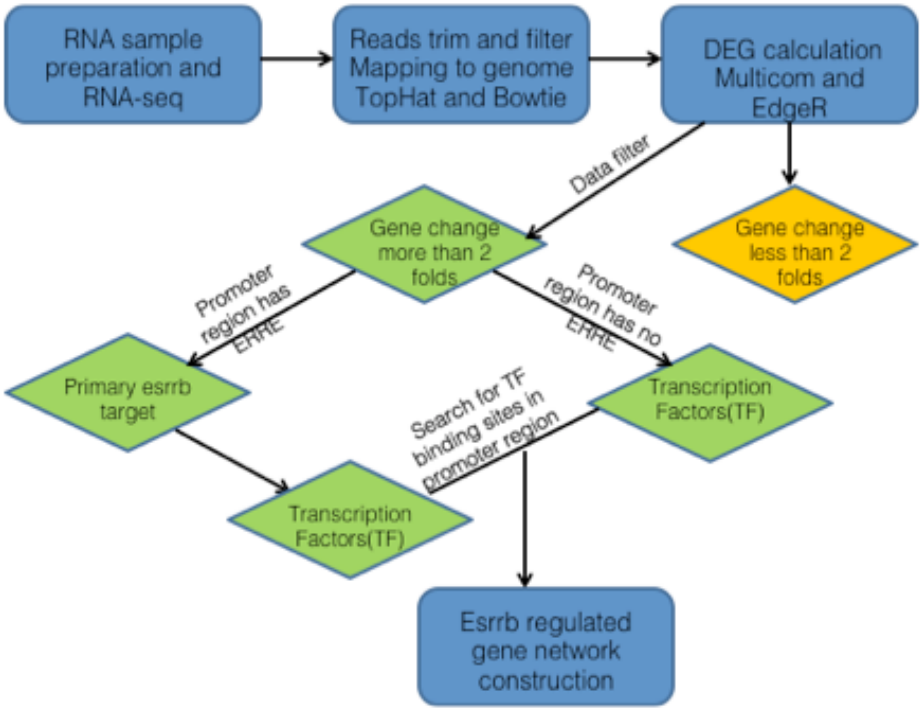


Figure II-7. Flow chart of gene regulatory network construction.

Differentially expressed genes are characterized by EdgeR (logFold Change (FC)<-1 or LogFC>1, FDR<0.05). Transcription factors in DEG list were retrieved by using Animal Transcription Factor Database. For every transcription factor, the upstream flanking region sequence were retrieved using R/BiomaRt (2.18.0), while transcription factor DNA binding motif was retrieved using R/MotifDb (1.4.0). The transcription regulation network was constructed by query the binding motif against differential expressed transcription factor upstream flanking region sequence.

a

Name	FC	Name	FC
GOS2	3.32	GALE	0.41
CDKN1A(p21)	2.10	PLD3	0.41
HOXB5	2.04	FDXR	0.40
CCNA1	0.50	LSS	0.39
GSN	0.49	SCP2	0.39
PFKL	0.48	PEX7	0.36
ASL	0.45	ACADS	0.36
IGFBP6	0.44	IL11RA	0.34
ILVBL	0.43	S100A4	0.32
IFI35	0.43	APLP1	0.26
MAN2B1	0.43	CEBPD	0.24
PCCB	0.42	EGR1	0.16

b

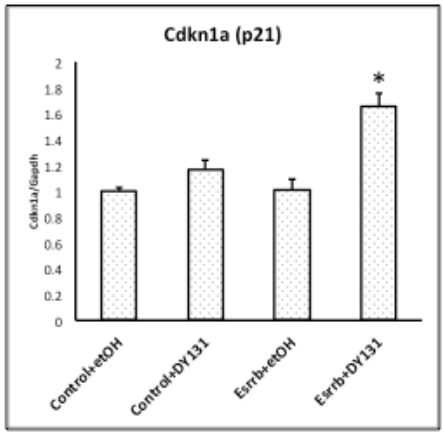


Figure II-8. DY131-activated Esrrb regulates known p53 target gene expression

(a) List of DY131-altered mRNAs in the presence of Esrrb, that are also known transcriptional targets of p53. (b) qPCR analysis was performed to confirm p21 mRNA concentration regulation requires both Esrrb expression and DY131 treatment.

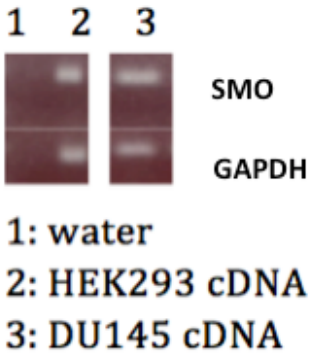


Figure II-9. RT-PCR of Smoothened in HEK293 and DU145 cells.

1000ng of HEK293 and DU145 total RNA was converted to cDNA library by reverse transcription using random primers and OligoTs. Smoothened and GAPDH transcripts were measured by PCR.

	Esrrb up-regulated (5 genes)	Esrrb down-regulated (10 genes)
DY131 up-regulated (6 genes)	*Agonist:*(0)	*Antagonist:* WDR52, F13A1, PXDN, SPNS2, LOC100506599, TAGLN (6)
DY131 down-regulated (9 genes)	*Antagonist:* LOC441454, TKTL1, SEMA3F, ZCWPW2, SDC2 (5)	*Agonist:* RARRES3, OASL, PADI2, DDX60 (4)

Figure II-10. DY131 is agonist/ antagonist for 15 mRNAs.

15 DY131 modulated Esrrb altered mRNAs are sub-grouped into 4 different groups based on each mRNA's behavior in response to Esrrb expression and DY131 treatment. The agonist is defined as DY131 further enhance Esrrb's effect, while antagonist indicates DY131 offset the effect of Esrrb.

Table II-1 Esrrb altered mRNAs (on-line/CD dataset)

Table II-2 Gene Ontology analysis of Esrrb altered mRNAs (on-line/CD dataset)

Table II-3 DY131 altered mRNAs when Esrrb is expressed (on-line/CD dataset)

Table II-4 Gene Ontology analysis of Esrrb-dependent DY131-altered genes (on-line/CD dataset)

Table II-5 Gene Ontology analysis results (on-line/CD dataset)

Table II-6 Esrrb expression with DY131 treatment (control vs. Esrrb+DY131) altered mRNAs (on-line/CD dataset)

CHAPTER III

Estrogen Related Receptor β Regulation of the Genes Targeted by The Hedgehog-Signaling Pathway

Abstract

Nuclear receptor family member, Estrogen Related Receptor β (Esrrb), and the hedgehog (Hh) signal transduction pathway are each reported to be significant for both tumorigenesis and induced pluripotent stem cell reprogramming. We hypothesize that Esrrb can modulate the Hh-signaling pathway and affect Hh-driven downstream gene expression. We established an Esrrb-expressing Hh-responsive NIH3T3 cell line and performed mRNA profiling using next generation sequencing (RNA-seq). Esrrb expression altered 171 genes (Log Fold Change<-1 or >1, p-adj<0.05), while Hh-signaling activation alone altered 339 genes (Log Fold Change<-1 or >1, p-adj<0.05), with an overlapping subset of 28 genes being altered by either. 12 of the 28 genes are changed in the same direction, including Tagln, Igf1, and Pfkfb3. In addition, RNA-seq showed that Esrrb expression in combination with Hh-signaling activation affects a group of 109 Hh responsive mRNAs, including Hsd11b1, Ogn, Smoc2, Igf1, Pdcd4, Igfbp4, Stmn1, Hp, Hoxd8, Top2a, Tubb4b, Sfrp2, Saa3, Prl2c3 and Dpt. The co-treatment of Hh ligand and DY131, which is an Esrrb ligand, can further alter the concentration of Hh differentially responsive genes in an Esrrb-dependent way. Interestingly, besides the well-known Gli transcription factor, we also found other important transcription factors, like Egr3 and Hoxd3, that can potentially regulate Gli independent transcription and serve as mediators for Hh response. **Conclusion:** Esrrb expression can regulate Hh-signaling driven genes. Our results suggest a new level of regulation of Hh-signaling by Esrrb, and potentially modulation of Esrrb by a small molecule ligand, like DY131, could be a new strategy to regulate various functions driven by the Hh-signaling pathway.

Introduction

Hh-signaling is a pivotal signaling pathway in embryonic pattern formation and stem cell/ cancer stem cell self-renewal as well as iPS induction [10, 76, 104, 106, 120, 121, 132, 136, 139, 159, 190, 281]. The pathway consists of the Hh membrane receptor Patched (Ptch), and the G-protein coupled receptor homolog Smoothened (Smo). When there is no ligand bound to Ptch, Ptch inhibits Smo and keeps the downstream pathway shut down. When Ptch binds to Hh peptide, the inhibition of Ptch on Smo is relieved and the Hh-signaling pathway is turned on. One of the broadly accepted mechanisms for Hh-signaling target genes response is through the binding of Gli family transcription factors to Gli-binding sites in the regulatory sequence of Hh regulated genes, which include the previously reported Gli1, Ptch1, Ptch2, Hhip1 genes [113, 141, 216, 228], as well as the recently discovered Hsd11b1 gene in fetal prostate [273] (Table III-1).

Hh-signaling is an essential pathway for embryogenesis, bilateral symmetry development, tissue homeostasis and tumorigenesis. An early study showed the Hh-signaling inhibitor, cyclopamine, is enriched in *Veratrum californicum.* This plant when consumed by pregnant sheep resulted in a midline differentiation defect in offspring [7, 115, 126, 131, 276]. Hh-signaling is important for reprogramming by driving the expression of Bim1, and the endogenous Smo activator, oxysterol, can facilitate reprogramming [159].

Similar to other core development related pathways, deregulated Hh-signaling due to the mutation or overexpression of pathway components and/or pathway ligand

induces a variety of types of cancers, which include basal cell carcinoma, medulloblastoma, bladder cancer, breast cancer, cervical cancer, liver cancer, colon cancer, prostate cancer, gastric cancer, pancreas cancer, head and neck cancer, lymphoma and non-small cell lung cancer [43, 55, 67, 71, 91, 100, 112, 138, 147, 161, 197, 227, 247, 263, 268]. The pivotal role of Hh-signaling in cancer development makes Hh-signaling an attractive target for drug development [52, 84, 149, 202]. For example, the FDA in 2012 approved GDC-0449, an Hh pathway inhibitor targeting Smo for basal cell carcinoma treatment [4, 52, 192].

Esrrb belongs to the nuclear receptor family [33, 82, 183]. It is important in early embryo development as genomic knock out of Esrrb is embryonic lethal due to the placenta deformation resulting from early differentiation of trophoblast stem cells [137]. Recent research showed that Esrrb was found to be a core reprogramming factor in inducing pluripotent stem cells (iPSC). C-myc and klf4 of OSKM (Oct4, Sox2, Klf4, c-Myc) can be replaced by Esrrb [70, 173, 229, 230]. Esrrb was also reported to drive Sox2 transcription to induce reprogramming in a single cell reprogramming system, revealing its central role in differentiation [19]. Esrrb has also been found to be important in tumorigenesis in both *in vitro* and *in vivo* studies. It is down regulated in prostate cancer progression and re-expression of Esrrb in prostate cancer cells inhibits cancer cell proliferation through tumor suppressor Cdkn1a/p21 induction [28, 274].

Interestingly, knocking down Esrrb in mouse embryonic stem cells was shown to affect the transcription of several Hh-signaling pathway related genes, including Gli2

and several Wnt family members, indicating potential regulation of Esrrb on Hh driven gene expression [172].

Though Gli transcription factors are relatively well known for transmitting Hh-signaling to target gene transcription, other factors mediating the Hh-signaling activity are not well studied. For example, Dner, Fbn2, Hsd11b1 and Brak are Hh responsive genes in fetal prostate, but overexpression of Gli1 or Gli2 cannot affect the transcription of these 4 genes. However, the expression of active Smo significantly increased the mRNA concentration of these genes [273], indicating there is at least one other mechanism accounting for the Hh-signaling target gene transcription regulation.

Due to the importance of both Esrrb and Hh-signaling in development and tumorigenesis, we hypothesize that Esrrb can regulate Hh-mediated transcription regulation and can serve as a regulator of Hh-signaling target genes, and thus would be a potential treatment target for Hh-signaling driven diseases.

Material and Method

Conditional Medium

Sonic Hedgehog Conditioned Medium (Hh-CM) is collected from cultured HEK293 cells carrying Sonic Hedgehog (Shh) N-terminus transgene, which was a gift from Dr. Phillip Beachy. Briefly, the Shh stable-transfected cells were kept in DMEM with 10% FBS until confluent, then the medium was switched to DMEM with 0.2%

FBS, the medium enriched with Shh-N terminus was collected 24 hours later, and was then filtered through 0.22uM filter, and stored in -80 degree freezer [112].

Cell lines and Expression vectors

Mouse embryonic fibroblast cells NIH3T3 were obtained from American Type Culture Collection (ATCC, Cat. No. CRL-1658). The cells are cultured in Eagle's Minimal Essential Medium (DMEM) (Invitrogen, Grand Island, NY) supplement with 10% Newborn Calf Serum (NBCS). The cells were transfected with either pcDNA3.1 (Zeo+) empty vector (Promega, Madison, WI) as control, or pcDNA3.1 (Zeo+)-Esrrb expression vector using Fugene HD (Promega, Madison, WI) according to manufacture's manual, and were further selected by supplement 150ug/ml Zeocine (Invitrogen, Grand Island, NY) to the culture medium for three weeks. Established cells were cultured until confluent, and are treated with 1:100 diluted Hh-CM for 48 hours, in phenol-red free DMEM supplement with 5% NBCS.

Reverse Transcriptase PCR and Real time PCR:

Total RNA was isolated and purified from NIH3T3-pc3.1 and NIH3T3-Esrrb using RNeasy kit (Qiagen, Venlo, Netherlands). 1000ng of total RNA was used to create cDNA libraries using Superscript III Reverse Transciptase (Invitrogen, Grand Island, NY) with random primers and oligodT. Quantitative PCR (qPCR) assays were carried out using SYBR GREEN qPCR using iQ supermix (BioRad, Herculus, CA) on ABI7500 system (Applied Biosytems, Foster City, CA). qPCR condition: 95 degree, 30 seconds; 60 degree, 40 seconds; 72 degree, 40 seconds. Primer sequences:

GAPDH (NM_008084), forward primer: AGCCTCGTCCCGTAGACAAAAT,
reverse primer: CCGTGAGTGGAGTCATACTGGA;
Patched (NM_008957), forward primer: CTCTGGAGCAGATTTCCAAGG,
reverse primer: TGCCGCAGTTCTTTTGAATG;
Gli1 (NM_010296), forward primer: GGAAGTCCTATTCACGCCTTGA,
reverse primer: CAACCTTCTTGCTCACACATGTAAG;
Igf1 (NM_001111274), forward primer: TGAGTGGCTTCCCTTGGGGG,
reverse primer: AGGTGTTGTTTTGTGGGTGGGGT;
Smoc2 (NM_022315), forward primer: GGAAGGAGCAGGGAAAGCAGATGAT,
reverse primer: TGGGCTGCTTGGCTTCCTCAAG;
Pdcd4 (NM_001168491), forward primer: GGACACTCCTAGGGCACCGC,
reverse primer: TCCGCTTCCCGCCTTTGGAC;
Stmn1 (NM_019641), forward primer: TCGGACCGAGCAGGGCTTTC,
reverse primer: CCGAGGGCTGAGAATCAGCTCAA;
Hp (NM_017370), forward primer: GAGGCAGTGTGTGGGAAGCCC,
reverse primer: GGTCAGCAGCCACTGGTCACT;
Ogn (NM_008760), forward primer: ACGACCTGGAATCTGTGCCTCC,
reverse primer: TTGGATTGCCCTCCAGGCGA;
Hoxd8 (NM_008276), forward primer: TTCCCTGGATGAGACCACAAGCAGC,
reverse primer: GTCTCTCCGTGAGGGCCAGAGT
Dpt (NM_019759), forward primer: TCAGTGCTGGATCGTGAGTGGC,
reverse primer: ACTGGCGATCCCTTTCCACTGC;
Top2a (NM_011623), forward primer: CCCAGGGAAGCTCCATGTCGG,

reverse primer: GGTTCCCTTTGGCGCAGCTC;

Igfbp4 (NM_010517), forward primer: GATCGTGGGGACACCTCGGG,

reverse primer: GCGGGGTGACACTGTTTGGGG;

Tubb4b (NM_146116), forward primer: TGTTGGCAGAGCGTCGGTTGT,

reverse primer: CGCTGATTACCTCCCAGAACTTGGC;

Hsd11b1 (NM_001044751): forward primer: CTGCCTGCCTGGGAGGTTGT,

reverse primer: TCCCTGGAGCATTTCTGGTCTGAAC;

Sfrp2 (NM_009144): forward primer: GGCCACAGAGGAAGCTCCCAA,

reverse primer: TCGGACACGCCGTTCAGCTT;

Saa3 (NM_011315): forward primer: ACAGCCAAAGATGGGTCCAGTTCA,

reverse primer: ACAGCCTCTCTGGCATCGCTGA;

Prl2c3 (NM_011118): forward primer: AGCCAGGCTCACACACTATGCAG,

reverse primer: CCCGTTCCGGACTGCGTTGA;

Immunoblot

600,000 cells were plated in 6-well-plate. After 24 hours growth in growth medium supplement with 10% NBCS, the cells were treated with 1% Hh-CM in phenol red free DMEM with 5% NBCS for 48 hours. Cells were lysed by protein sample buffer (BioRad, Herculus, CA) and boiled for 5min at 95 degree. 20ug of total protein was run on 12% SDS-PAGE gel. The proteins were then transferred to nitrocellulose membrane. The membrane was block by Phosphate buffered saline (PBS) with 0.05% Tween 20, 0.015g/ml dry milk and 0.015g/ml BSA. The membrane was then incubated with 1:2000 diluted monoclonal anti-Gli1 mouse IgG (Cell signaling, Beverly, Massachusetts,

Cat.No: L42B10), 1:2000 diluted monoclonal anti-Esrrb mouse IgG (R&D system, Cat. No: PP-H6705-00) and 1:2000 diluted polyclonal anti-GPADH rabbit IgG (Santa Cruz, Dallas, TX, Cat. No: sc-25777) overnight, washed, incubated with secondary antibodies diluted in PBS with 0.01g/ml BSA. The chemoluminescence was generated by west-Dura (Promega, Madison, WI) and recorded by X-ray film (Fisher Scientific, Pittsburg, PA).

Deep sequencing and data analysis:

2500ng total RNA from 2 biological replicates of each culture condition was extracted and purified, and then used to generate sequencing cDNA libraries using TruSeq Stranded mRNA Sample Preparation kits (Illumina, San Diego, CA) according to manufacturer's instruction. 8 samples were pooled in one lane and each sample was ligated to one specific barcoded aligner. cDNA library quality was determined by University of Missouri DNA core. The deep sequencing was performed by MU DNA core using Illumina HiSeq 2000. Around 18 million reads were generated in .fastq format. The sequencing reads were trimmed and filtered using FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit), and mapped to UCSC mm9 genome using Bowtie and TopHat [122, 237]. The resulting binary file (.bam file) for each condition is submitted to Galaxy public server (galaxy.psu.edu) for gene expression value quantification. Gene expression value is represented as Reads Per Kilobase of transcript per Million mapped reads (RPKM) by Cufflink reference genome guided transcript assemble. A gene model from different experiment was merged together by Cuffmerge and all pair-wise compassions of relative mRNA concentrations were analyzed by

Cuffdiff. Differentially expressed genes are determined by False Discovery Rate adjusted p-value (q-value) less than 0.05 ($q<0.05$).

Gene sorting and Hh differentially response genes characterization

All pairwise comparisons, control vs. Hh-CM, control vs. Esrrb, control vs. Esrrb + Hh-CM, Hh-CM vs. Esrrb + Hh-CM, Esrrb vs. Esrrb + Hh-CM, but not Esrrb versus Hh-CM treatment, were made through differentially expressed gene analysis Cuffdiff, and test results are stored for each transcript (Yes (Y): $q<0.05$, statistically significantly different; No (N): $q>0.05$, not significant). Depending on whether genes can pass certain pairwise differentially expression tests, each gene contains a matrix of the results from the 5 gene differential expression test and is sorted into 1 of the 32 groups using a in house R script (available upon request, Figure III-5). For each of the 16 groups of Hh responsive genes (control vs. Hh-CM, $q<0.05$), a logic determination is made to filter out the groups that have pairwise comparison results against themselves (group #6, #7, #8, #12, #13, #15, #16) or groups with no real world interest (#11). Leaving genes in groups #1, #2, #3, #4, #5, #9, #10 and #14 for further data filter and analysis. For the genes that passed the Hh-CM vs. Esrrb + Hh-CM test, we further filtered out genes that have $-0.5<[(RPKM_{(Esrrb+Hh\text{-}CM)} - RPKM_{Hh\text{-}CM})/ RPKM_{Hh\text{-}CM}]<0.5$, $-0.5<[(RPKM_{(Hh\text{-}CM)} - RPKM_{control})/ RPKM_{control}]<0.5$, and $-5<(RPKM_{(Esrrb+Hh\text{-}CM)} - RPKM_{Hh\text{-}CM})<5$. For the genes that doesn't pass the Hh-CM vs. Esrrb + Hh-CM, the genes that have $-0.5<[(RPKM_{(Hh\text{-}CM)} - RPKM_{(control)})/ RPKM_{control}]<0.5$, $[(RPKM_{(Esrrb+Hh\text{-}CM)} - RPKM_{Hh\text{-}CM})/ RPKM_{Hh\text{-}CM}]>0.1$ or <-0.1, $-0.5<[(RPKM_{(Esrrb)} - RPKM_{(control)})/ RPKM_{control}]<0.5$ are filtered out. All genes that have passed the above test are classified

as Hh differentially response genes, and they respond to Hh-CM treatment differentially in the conditions with or without Esrrb expression.

Gene Ontology and KEGG pathway analysis

DAVID bioinformatics source 6.7 is used for Gene Ontology (GO) analysis and KEGG pathway analysis [98, 99]. The gene name of associated altered mRNAs from certain pairwise comparisons were submitted to DAVID server (http://david.abcc.ncifcrf.gov) and GO analysis were performed for Biological Process (BP). Minimum counts were set as default value (2 counts) and maximum EASE score (p-value) was set to 0.05. Same parameter was used for KEGG pathway analysis.

Hh-signaling related genes enrichment analysis

Previous published Hh-signaling related gene sets generated by deep sequencing or microarray were retrieved. Refer to Table III-1 for specific experiment conditions and Hh-signaling targeted gene set descriptions. Briefly, only mRNAs altered more than 2 fold are included. Up-regulated and down-regulated mRNAs are sub-grouped and mRNA associated sequence identification is all converted to gene symbol. Hh altered mRNAs in our gene set are compared to the previous reported mRNAs, and enrichment p-value is calculated as previously described [283].

Promoter sequence analysis

For Gli transcription factor and Glucocorticoid Receptor (GR, NR3C1), R/Bioconductor package MotfiDb(1.4.0) were used to retrieve the binding motif. Differentially

expressed genes upstream flanking region sequences were retrieved using BiomaRt(2.18.0) [62, 63]. Sequence comparisons between the transcription factor binding motif and flanking region sequences were carried out by R command.

Reporter assay

NIH3T3 cells are grown to 70% confluent in DMEM with 10% NBCS. Medium was switched to phenol red-free DMEM with 5%NBCS. 8XGli-Luciferase reporter (Gli-LR) and sv40-renilla control vector is transfected to cells using Fugene HD (Promega, Madison, WI) for 18 hours. Transfected cells are seeded to 96 well plate and indicated treatments are performed for 48 hours. Luciferase assays were carried out using Dual-luciferase assay (Promega, Madison, WI). Briefly, cells are lysed using passive lysis buffer and then transferred to assay plate for luciferase signal quantification by Biotek Synergy 2 (Winooski, VT).

Statistical analysis

Other than RNAseq result analysis, all other experiments are statistically analyzed by t-test. Spearman Ranking Correlation is analyzed using R (version 3.0.2). RPKM value for each mRNA from both biological replicate in each condition are collected for correlation. The resulted pairwise correlation coefficients are stored in a matrix and Hierarchical clustering is created by R/Bioconductor (version 2.13) package Heatplus 2. Each Spearman correlation coefficient is color transformed for data visualization. Hh differentially response mRNA concentrations measured by RNAseq and qPCR are correlated using Pearson method in R. Briefly, for each tested mRNA, qPCR tested

relative concentration is normalized by the concentration of internal control GAPDH, and further normalized to control condition. RNAseq tested RPKM values are normalized to the value from control. Correlation is then performed between the above two set of normalized expression values.

Result:

Establishment of model cell lines

The NIH3T3 cell line is Hh-responsive but Esrrb negative. To make the NIH3T3 cells Esrrb positive, we transfected with Esrrb expression vector and used the empty vector as a control. Esrrb expression was confirmed using western blot (Figure III-1 a). The control vector transfected NIH3T3 cells (NIH3T3-pc3.1) have no Esrrb expressed, thus this cell line provided a clean Esrrb background. In contrast, NIH3T3 cells transfected with Esrrb expression vector (NIH3T3-Esrrb) have significant increased Esrrb protein concentration. Compared to HEK293 cells, which have endogenous Esrrb expressed, the concentration of overexpressed Esrrb in NIH3T3 cells attained a physiological relevant concentration (Figure III-1 a).

Hedgehog signaling and Esrrb regulated genes

To comprehensively characterize Hh-signaling driving mRNA changes in our system, we performed RNA-seq analysis on the mRNA isolated from NIH3T3-pc3.1 cells treated with vehicle control or 1% Hh-CM. Hedgehog signaling pathway activation after Hh-CM treatment was confirmed by significantly increased concentration of Gli1 by westernblot (Figure III-1b). After the treatment, we distinguished a total of 339

altered mRNAs having more than 2 fold change from Hh-CM treatment, with 245 of them up-regulated and 94 down-regulated (Table III-3, Figure III-3 a). Using reported Hh-signaling target gene sets generated by activating the Hh-signaling pathway or Gli transcription factor overexpression/ knock-down in different model cell lines or tissues as reference, we compare them to our Hh regulated gene sets [69, 75, 93, 124, 145, 165, 267, 273]. For all of the non-redundant 1337 genes from previous reports, we found 49 genes (p value=1.68e-06) that overlapped and another 290 new Hh-signaling responsive genes (Figure III-2a). In silico analysis of upstream sequences of differentially expressed genes showed that 27 of the Hh-CM response genes have Gli transcription factor binding site GACCACCCA (Figure III-6).

In addition to the well-characterized Gli transcription factor Gli1 and Gli2, we also found additional 22 known transcription factors, 1 chromatin remodeling factor and 5 transcription co-factors regulated by Hh-signaling pathway (Figure III-7). Gene Ontology (GO) analysis showed responsive genes enriched in the categories of cell cycle progression and cell proliferation regulation and organelle fission, supporting the role of known Hh pathway cellular function in cell proliferation promotion (Table III-2).

We also surveyed the gene expression in NIH3T3-Esrrb without Hh-CM treatment and control cells. 171 genes are differential expressed more than 2 fold by Esrrb (Table III-1, Figure III-4 a). We compared Esrrb-regulated genes to Hh-induced genes, and found there are 12 genes, Fabp4, Phex, Ccl5, Tagln, Aldh1a7, Lmod1, Cesla, Igf1, Mafb, Steap4, Pfkfb3 and Hlf, which are altered in the same direction by either Hh

ligand or Esrrb expression (Figure III-2 b, Figure III-5, #1 to #8). The presence of these Hh- and Essrb-regulated genes shows that Hh-signaling and Esrrb have functional overlaps in the regulation of these genes.

Hh-signaling regulated genes in the presence of Esrrb

Next we treated NIH3T3-Esrrb cells with Hh-CM and performed RNAseq analysis. Esrrb expression with Hh-CM treatment led to the most altered mRNAs compared to the control, revealed by the number of outlier genes in the scatter plot matrix (Figure III-3 a). Spearman ranking correlation of all mRNA profiles in 4 different conditions showed that each condition generates different gene expression revealed by the Spearman correlation coefficient, and correlation between Hh-signaling pathway stimulation in the presence of Esrrb expression and the control resulted in the lowest correlation coefficient in all pairwise comparisons (Figure III-3 b).

Using R script assisted gene sorting (Material and Method, Figure III-5), the genes of the most interest were found in groups #1, #2, #3, #4, #5, #9, #10 and #14 (Table III-4). These genes indicate Esrrb can regulate Hh-signaling pathway activity. Using the indicated filters of fold change and RPKM value described in Method and Material, we classified 109 genes that differentially respond to Hh-CM treatment when Esrrb is expressed (Figure III-5). We confirmed the concentration of 15 highly expressed mRNAs (saa3, prl2c3, dpt, sfrp2, pdcd4, smoc2, igf1, stmn1, top2a, tubb4b, hp, hoxd8, igfbp4, hsd11b1, ogn) by qPCR, with Pearson correlation coefficients between RNAseq and qPCR of at least 0.9 (Figure III-8). Among tested mRNAs, we found that when

Esrrb expressing cells are treated with Hh-CM, Sfrp2 (secreted frizzle related protein 2), Saa3 (serum amyloid A3), Prl2c3 (prolactin 2A3), Stmn1 (stathmin1), Hp (haptoglobin), Hoxd8 (homeoboxD8), Tubb4b (tubulin beta 4B), Top2a (topoisomerase II alpha) and Dpt (dermatopontin) have different mRNA concentrations compared to Hh-CM treatment alone in cells without Esrrb. These differences are more likely due to a proportional additive effect of Esrrb expression on altered baseline expression of the mRNAs (Figure III-4 a). In contrast, Igf1 (Insulin-like growth factor 1), Pdcd4 (programmed cell death 4) and Smoc2 (SPARC related calcium binding 2) lose their responsiveness to Hh stimuli when Esrrb is present (Figure III-4 b). On the other hand, Hsd11b1 (hydoxysteroid 11 beta dehydrogenase 1), Igfbp4 (insulin-like growth factor binding protein 4) and Ogn (osteoglycin) responded to Hh-CM better when combined with Esrrb expression (Figure III-4 b).

DY131 regulates Hh-responsive genes in an Esrrb-dependent manner

To test whether altering Esrrb activity by its ligand can alter the differential response to Hh-signaling, we also surveyed the effect of the Esrrb synthetic ligand DY131 on qPCR confirmed Hh differentially response genes [271, 286]. DY131 has been reported to be an Smo inhibitor by stimulating the removal of Smo out of primary cilia in the absence of Esrrb, thus demonstrating Esrrb-independent action of DY131 [255]. In the absence of Esrrb expression, we observed all of the 15 validated Hh differentially responding mRNA concentrations are inhibited at least 95% when DY131 and Hh were co-treated, confirming DY131 acting independently of Esrrb and supporting the role of DY131 as a Smo inhibitor independent of Esrrb (Figure III-4 a,

b). Similarly, when Esrrb is expressed with DY131, we found that the responses to Hh treatment of 6 of the 15 genes, Saa3, Igf1, Stmn1, Top2a, Tubb4b, Igfbp4 continued to be inhibited to Esrrb expression baseline, indicating the inhibition is Esrrb independent and likely through a Smo-inhibitory mechanism. In contrast, DY131 treatment cannot remove the effect of Esrrb on the Hh-CM response of 9 of the 15 genes, Prl2c3, Dpt, Sfrp2, Smoc2, Pdcd4, Hp, Hoxd8, Hsd11b1 and Ogn ($p<0.01$), showing Esrrb has an Esrrb ligand dependent activity in regulating these Hh-responsive genes. These data indicate that Esrrb ligands can alter Hh differentially responsive genes (Figure III-4 a, b).

Discussion

We distinguished 339 Hh-signaling altered mRNAs, among which are 48 known Hh-signaling target genes and 291 newly discovered targets (Figure III-3 a, Table III-3). When comparing Hh-driven genes to Esrrb-driven genes, the presence of 12 genes that changed the same way in response to either Hh-signaling or Esrrb expression strongly indicates Esrrb has functional overlap with Hh-signaling in transcription regulation (Figure III-2 b). The mRNAs under the regulation of either Hh-signaling or Esrrb include metabolic enzyme Pfkfb3 (6-phosphofructo-2-kinase/fructose-2, 6-biphosphatase 3), chemokine ligand Ccl5 (chemokine ligand 5), growth factor Igf1 (insulin-like growth factor 1) and cell migration/invasion related factor Tagln (transgelin), indicating both pathways regulate metabolism, cell migration and invasion, chemotaxis and cell proliferation.

Interestingly, we characterized 109 genes that behave differently in response to Hh ligand in the presence versus the absence of Esrrb expression. This group of genes contains genes like Igf1, which respond to Hh ligand treatment in the absence of Esrrb, but when Esrrb is expressed, Hh ligand treatment cannot modify its transcription (Figure III-4 b); genes like Hoxd8, which has differential expression in response to Hh stimulation when Esrrb is expressed, though this different response comes from the effect of Esrrb on basal level mRNA concentration. These genes indicate that Esrrb is upstream than Hh-signaling in regulating these genes (Figure III-4 a). We also observed genes like Hsd11b1 (CM vs. control: 21 fold; Esrrb+CM vs. Esrrb: 59 fold), Igfbp4 (CM vs. control: 3.4 fold; Esrrb+CM vs. Esrrb: 7.6 fold), and Ogn (Hh-CM vs. control: 57% inhibition; Esrrb+CM vs. Esrrb, 83% inhibition), which indicate that Esrrb and the Hh-signaling pathway synergistically regulate these genes (Figure III-4 b). These results demonstrated that Esrrb can regulate the Hh-signaling pathway.

The Esrrb synthetic ligand DY131 was shown to regulate Esrrb transactivation function in response element assays and be very specific in gene expression profile analysis in DY131-treated Esrrb-null or Esrrb-expressing cells [271, 286]. Coincidently, DY131, as well as its analog GSK4716, can move primary cilia located Smo out of its active subcellular compartment in an Esrrb-independent manner, drawing into question the Esrrb-specificity of DY131 [255]. This fact however does not eliminate DY131's ability to affect Hh-signaling driven mRNAs in an Esrrb-dependent manner.

DY131's effects were tested on 15 qPCR confirmed Hh differentially responsive mRNAs. Without Esrrb expression, the effects of Hh ligand treatment on almost all 15 tested genes are completely removed by DY131 treatment, confirming the role of DY131 as a Smo modulator independent of Esrrb. While when Esrrb is expressed, DY131's effect is different for Prl2c3, Dpt, Sfrp2, Pdcd4, Smoc, Hp, Hoxd8, Hsd11b1 and Ogn compare to the condition without Esrrb expressed (Figure III-4 a, b). Based on the observations, we conclude DY131 likely has two separate mechanisms that regulate the Hh-signaling pathway: one is Esrrb independent likely through Smo binding, while the other one is Esrrb dependent.

We suggest in the 100nM to 1uM concentration range, DY131 binds to Smo and induces its internalization without affecting Esrrb, ([255, 271, 286], Figure III-9). This inactivation of Smo leads to the inactivation of Smo-downstream signaling and shutdown of Smo-driven gene expression. As DY131 concentration increases into uM range, the Smo inhibitory effect is saturated and its activity as an Esrrb modulator begins to take place, which is consistent with the DY131 EC_{50} concentrations reported in the literature [271, 286]. When Esrrb is expressed, DY131 binds to both Smo and Esrrb and the overall gene expression observed comes from the net effect between the competition of Smo inhibition and Esrrb activity and we conclude that Esrrb has ligand dependent activity in regulating Hh-responsive genes transcription.

We also found 21 genes (group #25) that respond to Hh-signaling but only when Esrrb is present (Log2FC>1 or Log2FC<-1) (Figure III-10). 20 of them are up-regulated

and 1 is down-regulated. This indicates Esrrb has the ability to expand the transcription regulation of the Hh-signaling pathway.

In the canonical Hh-signaling pathway model, the activation of Smo transmits the Hh signal to Gli transcription factor through the activation and inactivation of several pathway components including Fused (Fu) and Suppressor of Fused (SuFu). We characterized a group of transcription related genes from the Hh responsive genes. In addition to Gli1, there are two transcription factors, Egr3 and Hoxd3, that have a Gli response element in their promoter region, and we presume they could be important factors in mediating Gli response on gene transcription (Figure III-6). However, the evidence that the transcription of Hsd11b1, Fbn2 and Brak respond to active Smo transfection, but not Gli1 or Gli2 overexpression, indicates that Smo activation has a Gli-independent function in regulating gene expression [273]. In support of this hypothesis, we found transcription factors that do not have a Gli-binding motif in their regulatory sequence, and these transcription factors could potentially initiate transcription regulation in parallel with Gli (Figure III-7).

We found Hsd11b1 is correlated to Hh-signaling activation from several reports in several model systems including fetal prostate, prostate cancer and embryonic fibroblast cell lines [160, 273], indicating cortisone converted from cortisol by Hsd11b1 may account for part of the Hh response gene profile. Surprisingly, we found the classic Glucocorticoid Receptor (GR) target gene, Mt2, along with 3 Hh-signaling activation inhibits all other genes that have Glucocorticoid Response Elements in their promoter

regions, Aldh1a7, Ankrd1 and Ism1 are repressed in response to Hh treatment (Figure III-11). Although Esrrb does not change the expression of Hsd11b1, Hh treatment with Esrrb expression further increased the mRNA concentration of Hsd11b1, accompanied by statistically significant alterations in concentrations of Mt2, Aldh1a7, Ankrd1 and Ism1 (Figure III-11). Our discovery strongly supports the idea that metabolite(s) downstream of Smo can also be mediators of Hh-signaling responses.

Interestingly, GR overexpression and the activation of its target genes are strongly associated with anti-androgen treatment in prostate cancer therapy. GR target genes overlap with those of the Androgen Receptor and have been determined to be involved in antiandrogen treatment enzalutamide resistance [5]. Inhibiting GR can restore enzalutamide sensitivity and Esrrb's activity in increasing Hsd11b1 indirectly represses GR activity by potentially lowering the GR ligand cortisol, and thus activating Esrrb may lead to better response of antiandrogen treatment and eliminate or postpone the resistance.

Our data provide useful reference marker genes for both Hh-signaling and Esrrb function. In addition, we also show that Esrrb has a role in the regulation of Hh-signaling driven genes. The mechanism of Hh-signaling is also expanded and a new layer of regulation of the Hh pathway through Esrrb is revealed, which may lead to improved treatments in diseases where Hh-signaling is important.

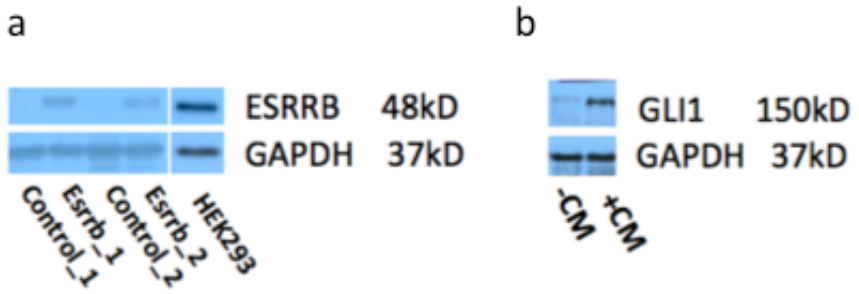
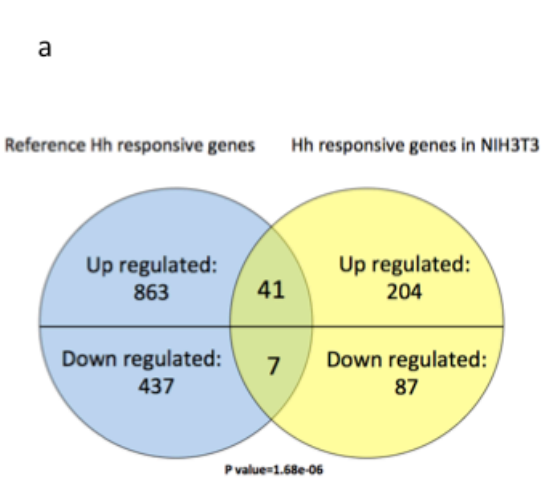


Figure III-1. Characterization of model cell line

(a) Expression of Esrrb is confirmed by Western blot. Two different Esrrb transfected cells showed successful expression of Esrrb protein. (b) 1% Hh-CM treated NIH3T3 transfected with empty vector showed increased concentration of Gli1 protein.



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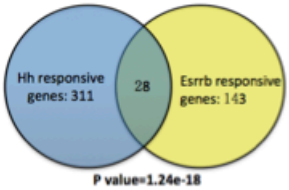


	Hh increased:	Hh decreased:
Essrb increased:	Igf1(16000) Mafb(16658) Steap4(117167) Pfkfb3(170768) Hlf(217082)	Adam12(11489) Kcnj15(16516) Sfrp2(20319) Stc1(20855) Dpt(56429) ABcc3(76408) Fam110c(104943) Npnt(114249) Prss35(244954) Slc36a2(246049) Zfp808(630579)
Esrrb decreased:	Inhba(16323) Slc40a1(53945) Serpinba1a(66222) Mfab4(76293) Pi15(94227)	Fabp4(11770) Phex(18675) Ccl5(20304) Tagln(21345) Aldh1a7(26358) Lmod1(93689) Ces1a(244595)

Figure III-2. Hh-signaling target genes

Previously reported Hh-signaling targeted genes were retrieved, only the genes that have more than 2 fold change are kept. The Hh responsive genes from our assay were compared to the reported genes. (a) Venn Diagram showing known Hh-signaling genes with the Hh-signaling responsive genes from our study were compared. The 48 overlapped genes (enrichment score = 1.68e-06) contain 41 Hh-signaling up-regulated genes, and 7 down-regulated genes. Hh responsive genes and Esrrb responsive genes were compared (b), and 28 genes (enrichment p value= 1.24e-18) were altered by either Hh-signaling activation or Esrrb expression. 12 genes among the 28 genes can be regulated the same direction by either Hh or Esrrb (Fabp4, Phex, Ccl5, Tagln, Aldn1a7, Lmod1, Ces1a, Igf1, Mafb, Steap4, Pfkfb3, Hlf).

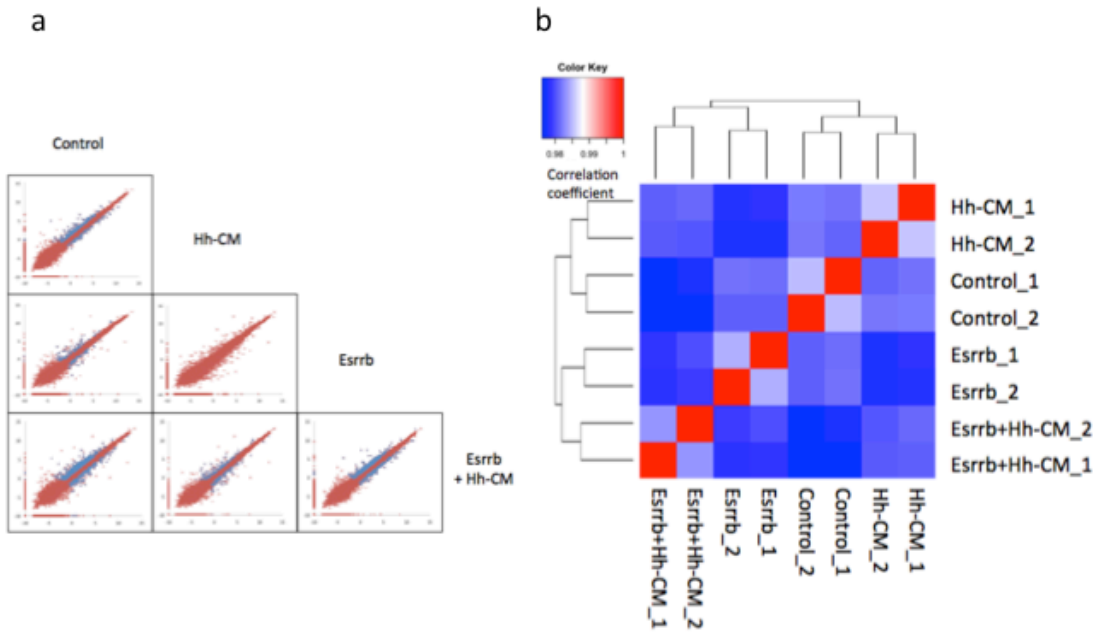


Figure III-3. Pair-wise comparisons for differentially expressed genes within Control, Hedgehog treatment, Esrrb expression and Esrrb expression plus Hedgehog treatment.

(a). Scatter plot of gene expression values in different conditions. 0.001 is added to the RPKM value of each transcript and resulted RPKM+0.001 values are log2 transformed. For each plot, each point ($X_{condition1}$, $Y_{condition2}$) on the plot represents the log2 (RPKM+0.001) of the gene in indicated conditions. If a certain gene passed the different expression test, that gene is highlighted by blue color, which if not differentially expressed, is red colored. All pair-wise comparisons are plotted, except Hedgehog vs Esrrb, which lacks biological meaning. (b). Spearman Ranking Correlation is used to analyze the similarity of mRNA profiles from control, Hh-signaling activation, Esrrb expression and Hh-signaling activation with Esrrb expression. Spearman ranking correlation coefficient is calculated and Hierarchical clustered. Correlation coefficient is color coded as indicated in the figure.

a

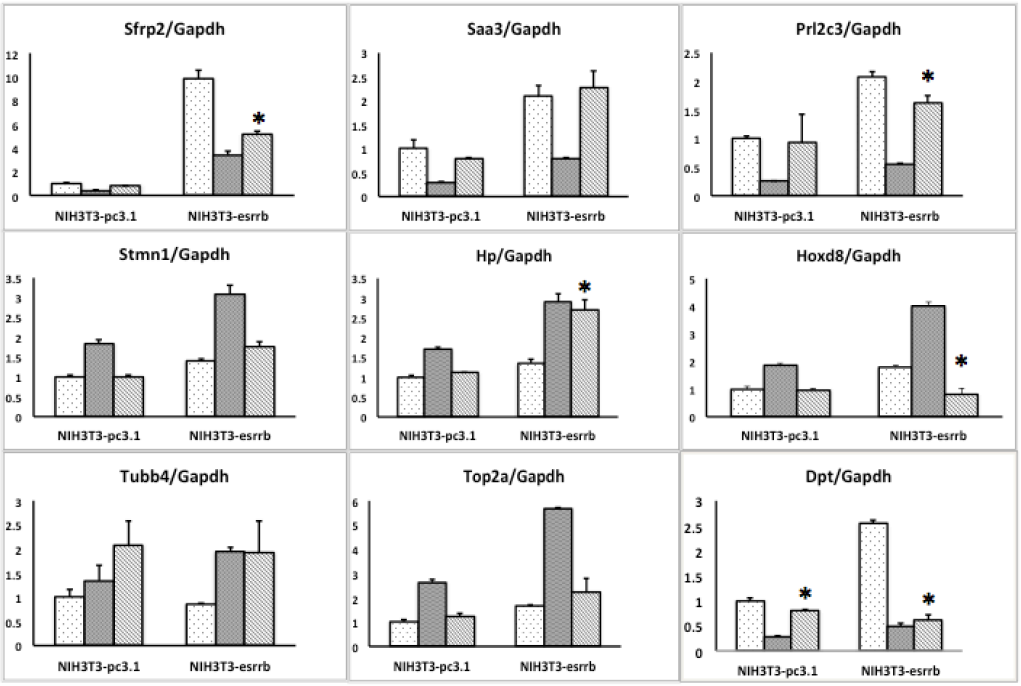


Figure III-4. Quantitative PCR validation of the top 15 Hedgehog signaling differentially responsive mRNAs.

Hh-signaling differentially responsive genes as determined by RNAseq were confirmed by qPCR after NIH3T3-pc3.1 and NIH3T3-Esrrb cells are co-treated with Hh-CM and 3uM DY131 for 48 hours. 109 of Effect of DY131 treatment is determined by comparing normalized mRNA concentration in Hh-CM+DY131 to no treatment control, p-values are calculated by t-test, *=$p<0.01$ compared to no treatment control in the conditions with or without Esrrb expressed. Each mRNA concentration is normalized to that of housekeeping gene GAPDH, and further normalized to the ratio in NIH3T3-pc3.1. (a) Esrrb altered the Hh-CM treatment response, due to a proportional additive effect from Esrrb expression.

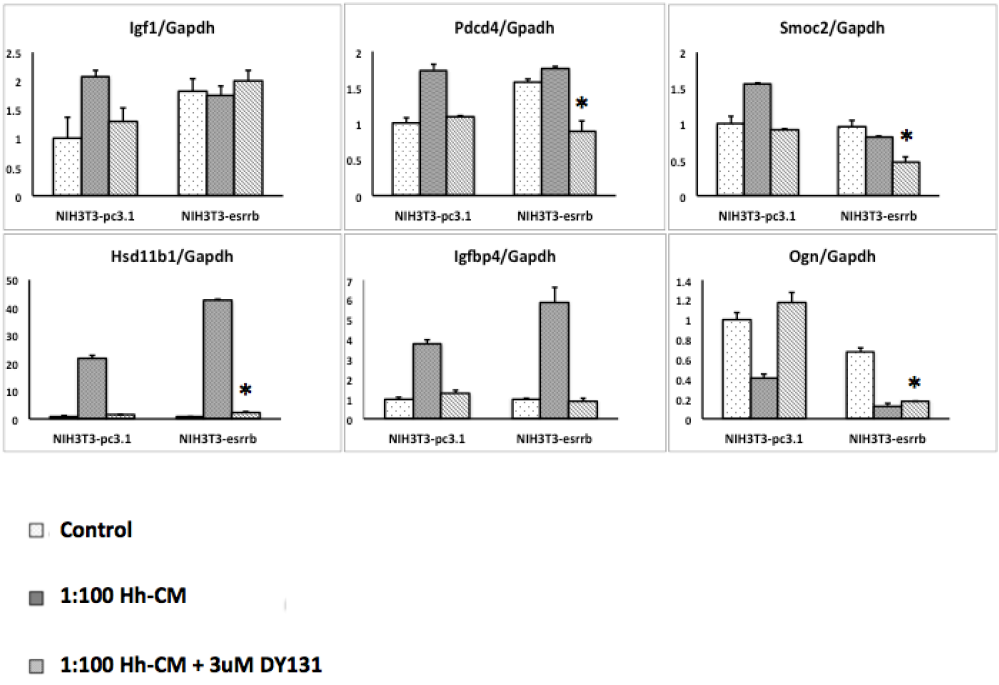


Figure III-4 (Continue)

(b) Genes respond to Hedgehog signaling better when Esrrb is expressed as well as genes losing Hh response when Esrrb is expressed.

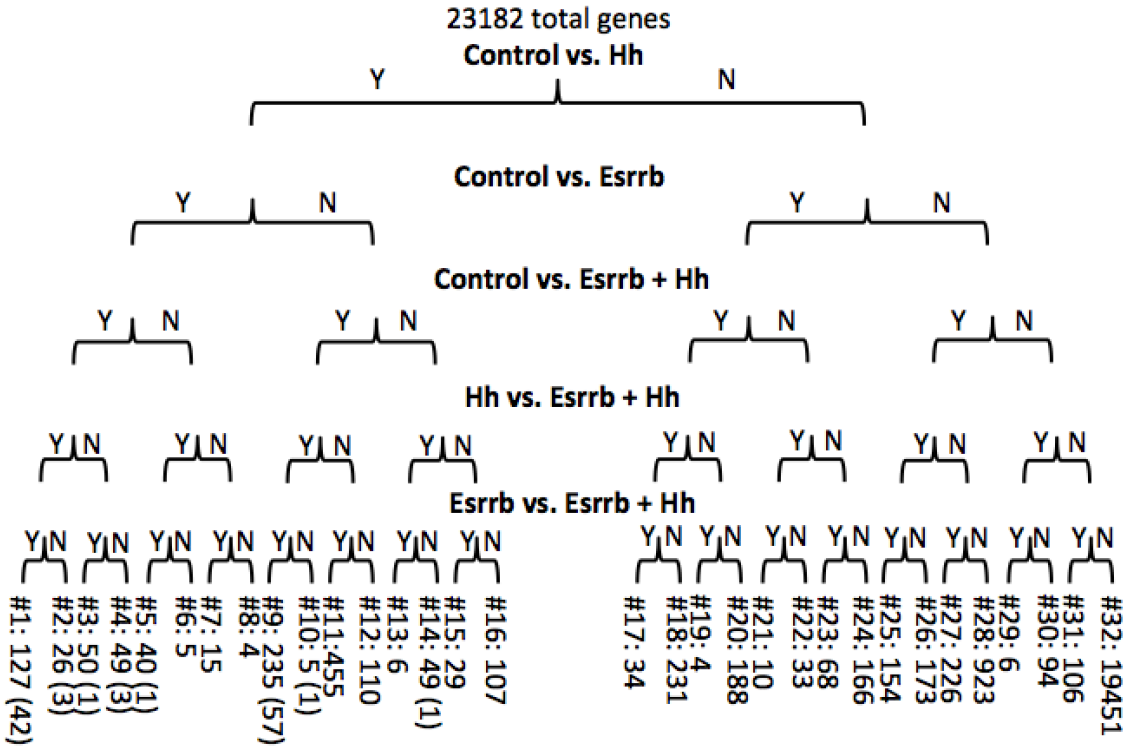


Figure III-5. Decision tree model of gene sorting

For all four biological conditions (control, Hh treatment, Esrrb expression, Esrrb expression with Hh treatment), all 5 possible but non-redundant pairwise comparisons for differentially expressed gene test were carried out. An array including all the transcripts each with the test results of the pairwise comparison was generated, and transcripts are grouped by whether they passed certain test using the “yes (Y)” or “no (N)” logic decision. A total of 32 groups were generated. Genes of interest for further analysis are subjected to further data filter based on fold change value and/or RPKM value.

No.	Gene ID	Gene Symbol	start	end	Sequence
1	17345	Mki67	-1502	-1489	GACCACCCATCCTC
2	14632	Gli1	-920	-907	GATCACCCACCGCG
3	13655	Egr3	-1958	-1945	GACCTCCCACGGCG
4	20203	S100b	-703	-690	CACCACCCACAATG
5	54611	Pde3a	-1400	-1387	GACCACCCCCACCC
6	19206	Ptch1	-3772	-3759	GACCACCCAAGTCG
7	66222	Serpinb1a	-1663	-1650	CACCACACACGAAG
8	244595	Ces1a	-116	-103	GACCACACATGGAT
9	12775	Ccr7	-1366	-1353	GGCCACCCACAGTC
			-1161	-1148	GACCACGCACCCAG
10	16000	Igf1	-1613	-1600	GACCACACAGAGGG
11	14528	Gch1	-275	-262	GGCCACCCAGGAGG
12	22137	Ttk	-1563	-1550	GACCACACAGTCTG
13	93689	Lmod1	-546	-533	GACCACCCAGTGAC
14	75600	Calml4	-998	-985	GACCACACACAGTG
15	18301	Fxyd5	-602	-589	GGCCACACACGCAG
16	74325	Cltb	-164	-151	GACCACCCAGCCCC
17	15366	Hmmr	-780	-767	GGCCACCCACGGCC
18	12615	Cenpa	-358	-345	CACCACCCACGTAA
19	16011	Igfbp5	-1970	-1957	GACCACCTATCATG
20	71145	Scara5	-1617	-1604	GACCACACACATCC
21	16765	Stmn1	-1979	-1966	GACCCCCCACCAGG
22	228421	Kif18a	-1361	-1348	GACCTCCCACCATC
23	15434	Hoxd3	-1648	-1635	GACCACTCACTTGG
24	66938	Sh3d21	-1961	-1948	GACCACACACTGGG
25	11499	Adam5	-15	-2	GACCACCAAGGGAG
26	11421	Ace	-1313	-1300	GCCCACCCACCCTC
			-685	-672	GACCACACATGATG
27	244954	Prss35	-1891	-1878	AACCACCCACTGCG

Figure III-6. Promoter Gli binding site analysis

Gene ID	Symbol	log2FC
Transcription Factor		
14283	Fosl1	-1.93
11910	Atf3	-1.67
630579	Zfp808	-1.23
13655	Egr3	-1.09
12394	Runx1	-1.05
71093	Atoh8	1.00
15434	Hoxd3	1.08
217082	Hlf	1.08
52679	E2f7	1.08
14235	Foxm1	1.09
55994	Smad9	1.11
108961	E2f8	1.13
15437	Hoxd8	1.16
16658	Mafb	1.19
16656	Hivep3	1.24
16600	Klf4	1.35
14633	Gli2	1.37
84653	Hes7	1.49
54139	Irf6	1.62
17301	Foxd2	1.71
22061	Trp63	1.87
207259	Zbtb7c	2.26
15205	Hes1	3.73
14632	Gli1	9.14
Chromatin Remodeling Factor		
12615	Cenpa	1.20
Transcription Co-factor		
12173	Bnc1	-1.59
107765	Ankrd1	-1.19
12189	Brca1	1.05
30937	Lmcd1	1.43
71648	Optn	1.63

Figure III-7. Hh-responsive transcription-related genes

Among the Hh-responsive genes, a group of transcription-related genes are discovered, including transcription factors, chromatin remodeling factors and transcriptional co-factors.

Gene	Control	Shh-CM	Esrrb	Esrrb+shh-CM	Correlation Score
Pdcd4	64.4	125.0	96.0	123.7	0.969
Tubb4b	58.1	89.2	77.0	159.2	0.940
Ogn	165.9	71.7	106.4	18.4	0.998
Dpt	77.5	17.0	160.4	35.3	0.994
Stmn1	36.0	72.1	49.3	116.8	0.998
Hp	41.5	62.6	40.3	113.0	0.981
Hoxd8	32.2	72.0	58.1	149.5	0.996
Top2a	15.0	44.3	21.8	99.5	0.998
Igfbp4	24.1	81.7	18.6	142.6	0.996
Igf1	3.5	9.8	7.5	10.2	0.896
Sfrp2	8.5	4.1	90.7	34.2	0.999
Hsd11b1	4.0	83.3	2.6	156.8	0.999
Saa3	1382.8	363.4	2632.8	893.4	0.996
Smoc2	78.0	119.5	63.3	63.8	0.978
Prl2c3	379.6	105.7	687.7	195.9	0.997

Figure III-8. Correlation of qPCR and RNA-seq of top 15 Hh differentially responsive genes

RPKM (Reads Per Kilo base per Million reads) values of qPCR-validated genes are normalized to NIH3T3-pc3.1. qPCR results from different conditions are also normalized to NIH3T3-pc3.1 condition. For each tested gene, both normalized RPKM values and normalized qPCR results are correlated by Pearson Correlation and correlation coefficients are shown.

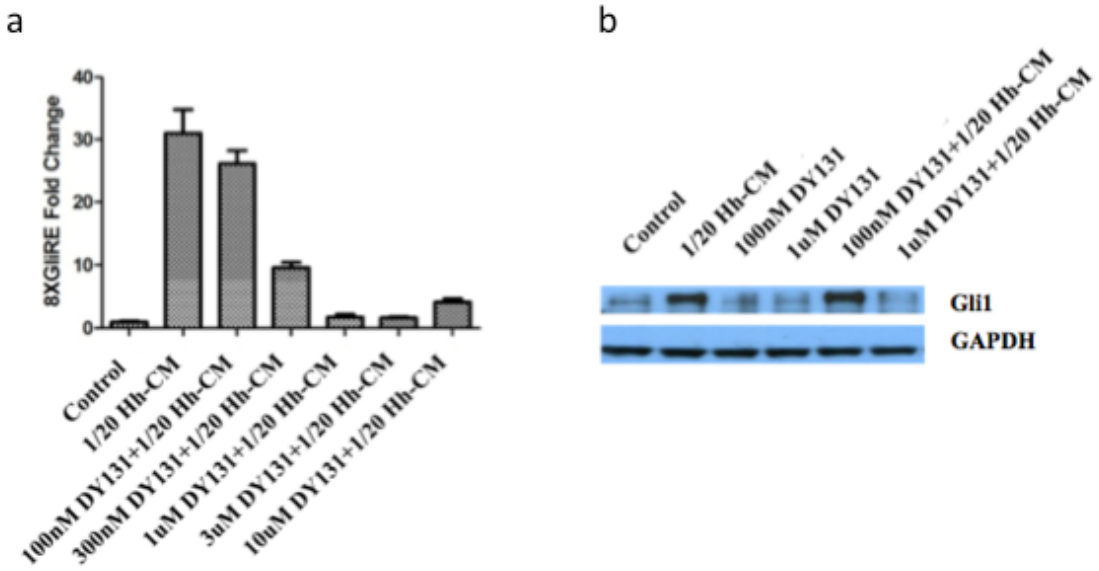


Figure III-9. DY131 is an Hh-signaling pathway inhibitor

(a) Using Gli response element, 100nM to 10uM DY131 with Hh-CM were co-treated in transfected cells. DY131 dose-dependently inhibited Hh-CM stimulated Gli luciferase activity. (b) Consistent with the reporter assay, 100nM DY131 cannot inhibit Hh-CM stimulated Gli1 protein concentration, while 1uM DY131 can inhibit the stimulation of Hh-CM on Gli1 protein concentration.

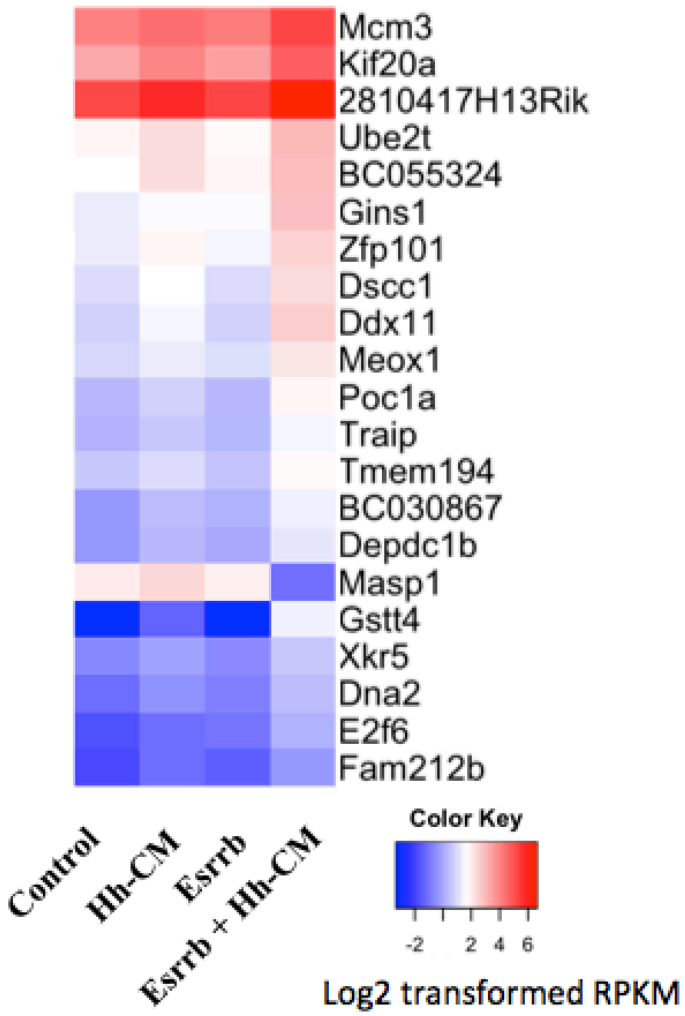


Figure III-10. Esrrb-dependent Hh-responsive genes

21 genes showed Hh response only when Esrrb is expressed (Control vs. Hh, q>0.05; Control vs. Esrrb, q>0.05; Control vs. Esrrb+Hh-CM, q<0.05, logFC>1 or <-1; Esrrb vs. Esrrb+Hh-CM, q<0.05, LogFC>1 or <-1; Hh vs. Esrrb+Hh-CM, q<0.05;). The averaged RPKM values of each gene is log2 transformed and further color transformed in heatmap.

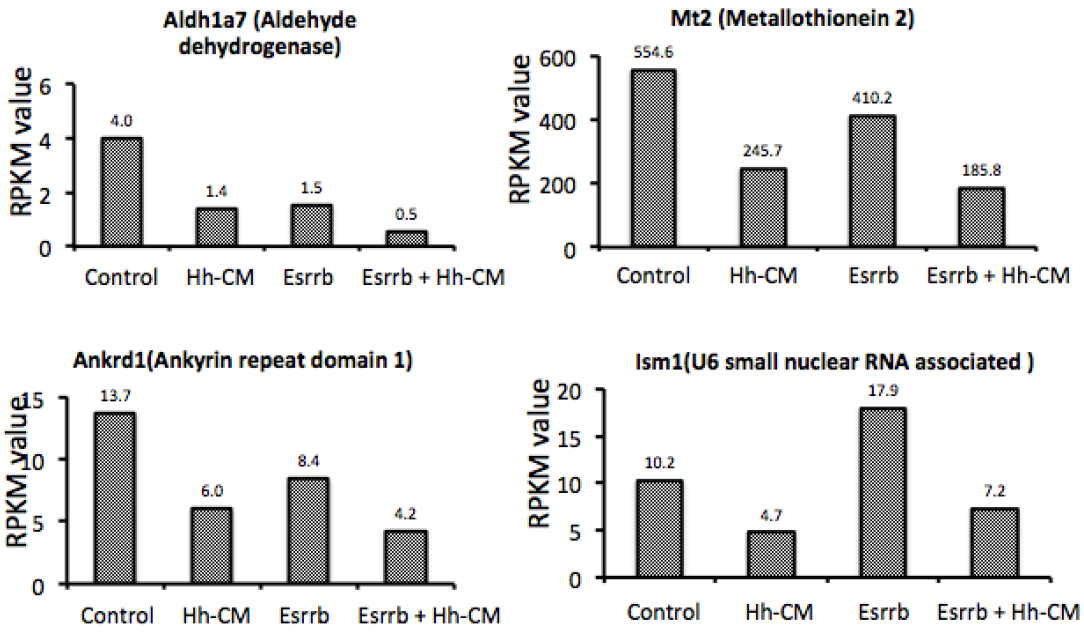


Figure III-11. Glucocorticoid Receptor related genes in response to Hh-CM and Esrrb

4 genes having Glucocorticoid Receptor Response Element in their promoter regions are enriched from Hh responsive genes. All four of them, when treated with Hh-CM, are repressed, showed by decreased RPKM value compared Hh to Control. When Hh-CM is treatment in the presence of Esrrb, the RPKM values of all 4 genes are further changed compared to Hh-CM treatment without Esrrb expressed.

CHAPTER IV

SMO, ESRRB AND DY131 ARE INDEPENDENT MODULATORS OF AKT ACTIVITY

Abstract:

Basal Cell Carcinoma cells experience rapid decreases in sensitivity to the FDA approved Hedgehog (Hh)-signaling inhibitor Vismodegib. Akt over-activation has been shown to be one of the mechanisms accounting for this resistance. We find in this chapter that activated Hh-signaling pathway can lead to Akt phosphorylation and stimulate its activity. We also found DY131 and Esrrb can inhibit Akt phosphorylation, although the DY131 effects are likely to be independent of both SMO and Esrrb. The ability of DY131 and Esrrb to inhibit Akt phosphorylation indicates both can potentially be utilized to both prevent Vismodegib resistance and inhibit growth in resistant tumors.

Introduction:

Hh-signaling pathway is a pivotal pathway in regulating cellular proliferation, development, wound healing and tissue homeostasis [103, 112, 182]. Loss of regulation of Hh-signaling pathway has shown to be a driving factor in many types of cancer. Compounds like Cyclopamine (Cyc) and GDC0449 (Vismodegib) that can inhibit Hh-signaling pathway by inhibiting Smoothened (SMO) can eliminate cancer in either pre-clinical studies or clinical application [112, 197]. Vismodegib was approved by FDA for autocrine Hh-signaling driven Basal Cell Carcinoma (BCC) treatment [6].

Although anti-Hh treatment offers promising effect on BCC treatment, resistance to Vismodegib is rapidly observed. Acquired resistance comes from SMO mutation, which disrupts Vismodegib binding to SMO and the over-activation of Akt and its downstream signaling [150, 189]. Due to these reasons, it is desirable to either find a

second-generation Hh-signaling inhibitor that can overcome Hh-inhibitor resistance, or to figure out a way to modify a resistance-driving factor to prevent the resistance from occurring.

Material and Method:

Cell culture and Transfection (refer to Chapter II and III Material and Method)

Immunoblot (refer to Chapter II and III Material and Method)

Results and Discussion:

Since Akt was shown to up-regulate Hh-signaling downstream transcription factor Gli activity, it is not surprising to see that over-activated Akt accounts for a mechanism for anti-Hh treatment resistance [198]. Also, Akt was shown to crosstalk to several G protein-coupled receptors, including SMO, and Akt activation is turned on when SMO is activated [189]. We hypothesize that activated SMO can lead to Akt phosphorylation and activation.

To test this hypothesis, we tested Akt phosphorylation concentration after Hh-CM treatment in NIH3T3 cells. In addition to the increase in Gli1 protein concentration, Hh-CM treatment also increases Akt phosphorylation at Serine 473 (pAkt Ser473), a marker for Akt activation [148](Figure IV-1).

In Chapter III, we showed that DY131 dose-dependently inhibits Hh ligand-activated Gli-reporter activity with an IC50 of 500nM. We also showed in Chapter III that DY131 inhibits all of the 15 tested Hh-signaling responsive genes independent of Esrrb, supporting the earlier report that DY131 binds to activated SMO and induces the internalization of SMO independent of Esrrb [255]. With the observation that Hh-treatment increases pAkt Ser473, we hypothesize DY131 can also lead to changes in the Hh-CM induced pAkt Ser473.

As mentioned in Chapter III, DY131 treatment alone has no effect on Gli1 protein concentration, while 1uM DY131 can completely inhibit the Hh-induced Gli1 protein concentration increases (Figure IV-1). In contrast to Gli1's response to DY131, 100nM DY131 treatment without Hh-CM slightly inhibits basal level pAkt Ser473. Additionally, 100nM DY131 co-treat with Hh ligand also generates similar inhibitory effect of Hh elevated pAkt (Figure IV-1). 1uM DY131 has larger effect in inhibiting basal level pAkt-Ser473. When 1uM DY131 is co-treated with Hh-CM, the Hh-CM induced Akt phosphorylation is completely removed. We conclude that DY131 can inhibit both basal or Hh-induced pAkt Ser473. Since NIH3T3 is Esrrb null, we also conclude this pAkt inhibition effect is Esrrb independent.

Additionally, although 100nM and 1uM DY131 treatments have no effect on Gli1 in the absence of Hh-CM, 100nM and 1uM DY131 slightly inhibit the basal pAkt Ser473 concentration. From this observation, we hypothesize that DY131's effect on pAkt-Ser473 is Hh-activated-SMO independent.

To further test this hypothesis, we treated DU145 cells, which is Esrrb null and does not have either SMO agonist (SAG) or Cyc responses (refer to Chapter V), with DY131 and measured pAkt Ser473 concentration. 1uM and 10uM DY131 dose-dependently inhibit pAkt Ser473 without affecting total Akt protein concentration (Figure IV-2). This result shows that the pAkt inhibition effect of DY131 is less likely to be a collateral effect of SMO inhibition, but more likely is an independent effect on Akt or other signaling pathways that lead to Akt phosphorylation. Combining the results regarding DY131 from Chapter I, II and III, we conclude that DY131 has at least 3 targets, Esrrb, SMO and Akt.

Very interestingly, when we overexpressed Esrrb in DU145 cells, we observed a similar decrease in Akt phosphorylation (Figure IV-3 a). In the Esrrb-expressed DU145 cells, we knocked down the overexpressed Esrrb and Esrrb's effect on pAkt Ser473 is removed (Figure IV-3 b). The reason that Akt phosphorylation does not return to a basal concentration is likely due to the not-100% knockdown of Esrrb. Akt phosphorylation is correlated to cellular proliferation. Although the specific mechanism of how Esrrb inhibits Akt phosphorylation requires further investigation, the result that Esrrb can inhibit Akt phosphorylation/ activity indicates that Esrrb can be a central factor regulating cellular proliferation.

One interesting observation from Chapter II is that DY131 alone without Esrrb expressed dose not change any mRNA concentration. This indicates that DY131's effect

is at the protein post-translational-modification level. Genome-wide Akt signaling of target genes is not well documented, so we cannot enrich the Esrrb target genes with Akt signaling. However, our gene ontology analysis of Esrrb target genes in Chapter II showed that genes involved in regulation of proliferation and apoptosis are the top hits, thus supporting the result of Akt phosphorylation inhibition by Esrrb.

We have clearly shown that Esrrb can regulate Hh-signaling target genes in Chapter III, and we concluded the regulation potentially took place downstream of SMO (Figure IV-4). Our results indicate that Akt could be a potential target of Esrrb involved in Hh-signaling target genes regulation.

In conclusion, DY131 is capable of inhibiting Hh-activated SMO as well as Akt phosphorylation. This advantage of DY131 over Vismodegib, which inhibit SMO but not Akt phosphorylation, indicates DY131 has the potential to be the second-generation Hh-signaling inhibitor with the ability to overcome SMO inhibitor resistance. The result that Esrrb can inhibit Akt phosphorylation also indicates Esrrb could be a significant factor in regulating cell proliferation, and manipulating Esrrb activity through an Esrrb ligand or targeting Esrrb downstream genes can potentially prevent the occurrence of current Hh-signaling inhibitor resistance.

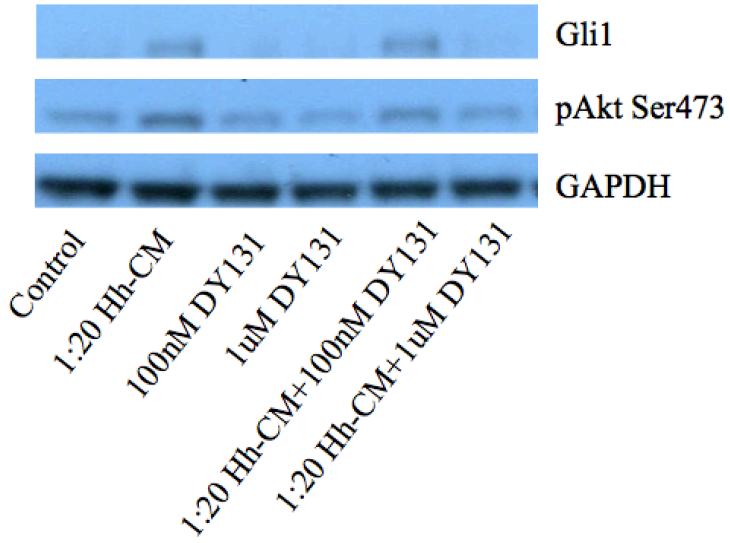


Figure IV-1 DY131 inhibits Hh-induced Akt phosphorylation

NIH3T3 cells are treated with 1 to 20 diluted Hh-CM, with or without 100nM or 1uM DY131 for 48 hours. The increased concentration of Gli1 protein is accompanied with Akt phosphorylation at Serine 473. 100nM DY131 has minimal effect on decreasing Hh-CM induced Gli1 protein, as well as inhibiting Hh-CM induced pAkt. 1uM DY131 is capable of inhibiting both Gli1 protein concentration and pAkt to basal level.

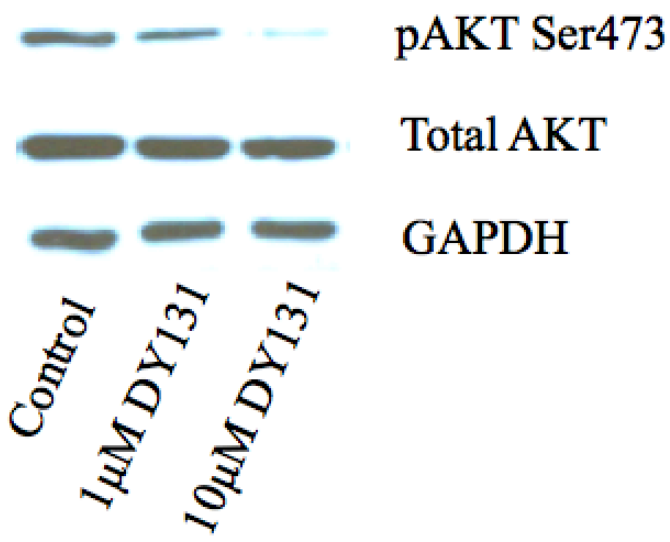
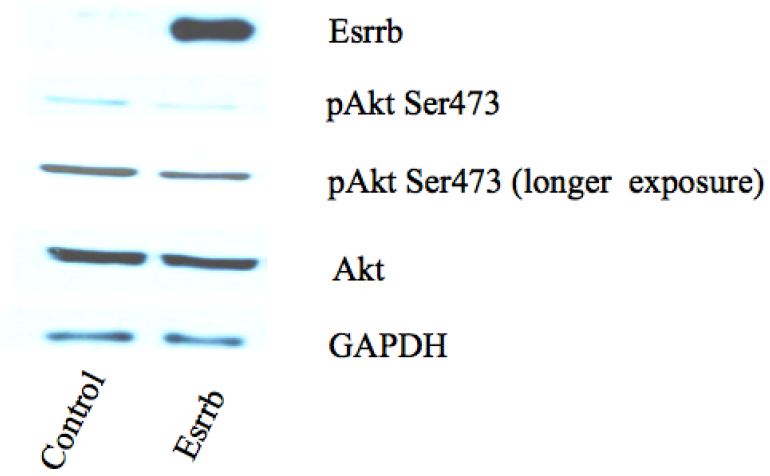


Figure IV-2 DY131 inhibits basal level Akt phosphorylation in DU145 cells

DU145 cells are treated with 1uM and 10uM DY131 for 24 hours. 1uM DY131 slightly inhibits Akt phosphorylation and 10uM DY131 completely inhibits Akt phosphorylation.

a



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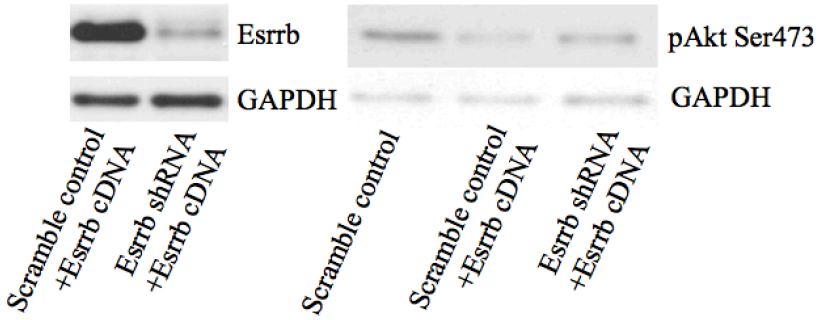


Figure IV-3 Esrrb inhibits basal level Akt phosphorylation in DU145 cells

(a) DU145 cells are transfected with either control vector or Esrrb. Expression of Esrrb inhibits Akt phosphorylation. (b) When Esrrb expressed DU145 cells are shRNA treated, shRNA against Esrrb can decrease the overexpressed Esrrb protein concentration. When overexpressed Esrrb is knocked down, Akt phosphorylation concentration increased.

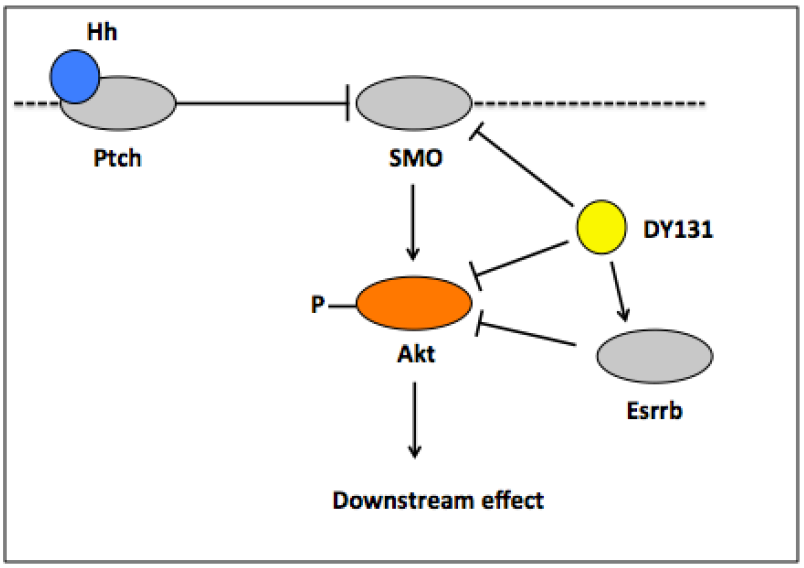


Figure IV-4 Illustration of SMO, DY131 and Esrrb's effect on Akt phosphorylation

CHAPTER V

SUMMARY AND PERSPECTIVES ON FUTURE RESEARCH

Summary:

As an important member of the orphan nuclear receptor family, Esrrb has been shown to have important roles in embryo development, reprogramming differentiated cells to (induced pluripotent stem) iPS cells and tumorigenesis [70, 137, 274]. However, very little attention has been paid to Esrrb for cancer research since early studies showed Esrrb is not expressed in adult tissues. Thus only a small portion of target genes regulated by Esrrb has been discovered.

In Chapter II of this dissertation, we reported our discovery of both ligand-independent and ligand-dependent Esrrb transcriptional targets in human metastatic prostate cancer cells. We distinguished a set of genes that carries (Steroid Factor Response Element) SFRE/ (Estrogen Related receptor Response Element) ERRE in their promoter regions, indicating they are the direct transcriptional targets of Esrrb.

In Chapter III, we reported other major objectives of this dissertation: to characterize the novel transcription target of Hh-signaling pathway, as well as to distinguish functional overlap between Hh-signaling pathway and Esrrb, and to discover the interference of Esrrb in regulating Hedgehog-signaling target genes.

In Chapter IV, we reported our discovery that either DY131 or Esrrb can independently inhibit Akt phosphorylation. The results in this chapter indicate both DY131 and Esrrb can be used to prevent the resistance of anti-Hh signaling treatment Vismodegib.

In addition, we applied gene expression profiling using next-generation sequencing to TRAMPC2 cells treated with Hh and/or Sutherlandia extract to characterize Sutherlandia function as well as to distinguish Sutherlandia response biomarkers (Appendix-I). We found that Sutherlandia extract contains components that strongly inhibit the Hh-signaling pathway.

Finally, we developed a bioinformatics tool by which a gene regulation network can be built based on matching transcription factor binding motif to coding gene upstream sequence (Chapter II). This tool can be used for *in silico* transcription factor direct target prediction, and also provides a method to direct future mechanism studies.

Along our research, we also distinguished a few interesting observations that worth further investigation. The preliminary data regarding the observations have potential to be fully developed to future projects. They are summarized below.

Common Esrrb target genes in both human and mouse cells

From Esrrb altered genes in both human and mouse cell lines in Chapter II and III, we used a known human-mouse ortholog database and found ortholog genes (http://www.genenames.org). These orthologs allow us to find 52 common Esrrb transcriptional targets, which can serve as markers for general Esrrb activity evaluation. Our promoter analysis revealed that 15 of the 52 common Esrrb responsive genes in both human and mouse cells have ERRE in the proximal promoter region, indicating the

transcription regulation of these genes can be modulated by Esrrb directly binding to the promoter and interacting with transcription machinery. Gene ontology analysis showed 11 genes are involved in transcription regulation, this reconfirms Esrrb is a master transcription factor. Among these 52 orthologs, Tagln, Aox1, Unc5a, Ifi44, Adamts15, Trim2, Brdt, Rasl11a, Ptipnc1 and Adam12 do not require DY131 to be altered by Esrrb, while the remaining 42 genes rely on DY131 binding to Esrrb to be altered (Figure V-1). Using these common Esrrb target genes, a small PCR array can be established to facilitate the screening for Esrrb ligands from compound libraries.

Transgelin (Tagln) is an important downstream factor of Esrrb

One of the Esrrb target gene, Tagln, was reported to be capable of inducing cell movement and metastatic behavior of the prostate cancer cells [123, 187]. The fact that Esrrb inhibit the expression of this gene indicates that Esrrb is an inhibitor of cancer cell movement and potentially can inhibit the metastasis of cancer cells. Tagln has two copies of ERRE in its promoter region. In human, one of them is more than 1000 nucleotides upstream of coding sequence, while another one is in the proximal promoter region (-192 to -181 nt), while in mouse, both ERREs are in the proximal promoter region, showing Tagln is a strong Esrrb transcriptional target (Figure V-2). Since this gene is also DY131 responsive when Esrrb is expressed, Tagln is a strong biomarker to reflect Esrrb's activity.

We labeled DU145-Esrrb cells that were established in Chapter II with Green Fluorescent Protein (GFP) label (DU145-Esrrb-GFP) and monitored whether Esrrb

expression alters the cancer cell-fibroblast cell interaction (refer to the next part of this chapter). Interestingly, when DU145-Esrrb-GFP cells are co-cultured with NIH3T3 cells, the labeled cancer cells formed sharp boundary colonies, compared to their counterparts with no Esrrb expressed, which formed rough boundary colonies (Figure V-5). We think the observed DU145 colony phenotypical change is due to the cell movement change: a rough colony boundary indicates active movement of DU145 cells, while a sharp colony indicates silenced movement of DU145 cells and no expansion of the cancer cell colony. We presume that this Esrrb associated morphological change is due to the altered expression of microenvironment related genes. We hypothesize repressed Tagln by Esrrb is the reason for the observed phenotypical alteration.

Construction of paracrine Hedgehog-signaling pathway in co-culture system

Interestingly, we discovered the autocrine Hh-signaling pathway in most prostate cancer cells, including DU145, PC3 and LnCaP, is missing and therefore lack a detectable Gli response to Hh-signaling pathway modulators. In our lab, we never observed Gli reporter activity response to Hh ligand-enriched Conditional Medium (Hh-CM), SMO agonist (SAG), or Hh-signaling inhibitor cyclopamine (cyc) in human prostate cancer cells (Figure V-3). On the other hand, proliferation in Hh-signaling responsive fibroblast cells is never observed to be related to Hh-signaling activation, questioning the relationship between Hh-signaling responsiveness and proliferation.

Although the human prostate cancer cells were reported to have Gli1 or Gli2 dependent proliferation, their Gli-dependent proliferation is more likely not mediated by

canonical Ptch-SMO-Gli axis, but through non-canonical pathways downstream of SMO [201, 206, 212]. However, when LnCaP cells were co-cultured with osteoblast cells, cyclopamine treatment successfully repressed the proliferation of LnCaP cells. This observation mimicked the effect that cyclopamine inhibits the proliferation of PC3 xenograft tumors *in vivo* [57, 112]. These results indicate the proliferation stimulation effect of Hh ligand as well as proliferation inhibitory effects of Hh-signaling inhibitors need to be processed by stromal cells. An acceptable model accounting for the observation is paracrine Hh-signaling pathway, in which stromal cells are Hh-signaling responsive and synthesize and secret a pre-proliferative factor to the microenvironment and this factor can stimulate the growth of tumor cells adjacent. This paracrine Hh-signaling model is supported by the studies reporting bone morphogenetic protein BMP4, as well as several different fibroblast growth factors are involved in this cancer cell-stromal cell paracrine Hh-signaling[57, 207].

To construct a paracrine Hh-signaling system to test whether the anti-proliferation effect of anti-Hh treatment is stromal cell dependent, we established a co-culture system by mixing Hh non-responsive prostate cancer DU145 cells with Hh-signaling responsive mouse embryonic fibroblast NIH3T3 cells. To distinguish the two types of cells in the culture, we stably transfected GFP into DU145 cells, and transfected Gli reporter and control Renilla reporter into NIH3T3 cells. We titrated the amount of DU145 cells co-cultured with NIH3T3 cells and measured Hh-signaling activity in fibroblast cells by either Gli reporter assay, or qPCR using mouse specific Ptch1 primers (Figure V-4). Both assays showed that co-culture with cancer cells increased Hh-signaling pathway

target gene Ptch1 as well as its Gli transcriptional activity, indicating Hh-signaling pathway in NIH3T3 cells is activated by co-culturing with DU145 cells. Under the confocal microscope, cancer cells, which are indicated by GFP signal, form colonies surrounded by fibroblast cells (Figure V-5). When exogenous Hh ligand was added to the co-culture system, we observed enlarged DU145 colonies and an increased number of DU145 cell colonies, indicating increased cancer cell proliferation (Figure V-5). By using this system, multiple compounds, including botanical extracts, can be tested for their potential cancer proliferation inhibition effects that are mediated by paracrine Hh-signaling.

Esrrb and cancer stem cells

Recent studies about stem cell and cancer stem cell showed that p53 tumor suppressor decreases the plasticity of cell type conversion from differentiated cells to stem cells [152]. We clearly observed that Esrrb, when stimulated by DY131, or stimulated by un-characterized endogenous ligand, could up-regulate the mRNA and protein concentration of cell cycle regulator p21, which is a well-known downstream factor of p53. We also saw a significant increase of p53 C-terminal phosphorylation, indicating Esrrb overexpression and activation is capable of up-regulating p53 activity.

As mentioned earlier in the dissertation, Esrrb is capable of reprogramming differentiated cells to pluripotent stem cells along with Oct4 and Sox2, by inducing core reprogramming factors [70]. Interestingly, another core reprogramming factor, c-myc, can also up-regulate p53 activity in stem cells reprogramming [178]. Studies have

shown that p53 knock-down can efficiently facilitate the establishment of reprogramming, and p53 knock-down generates very similar gene expression signature as stem cells [45, 154, 232]. These results indicate that p53 is a barrier for the reprogramming. From this perspective, we think the p53 activity induction effect of Esrrb and c-myc is a feedback effect of p53 to limit and suppress the reprogramming, although we don't know how much "reprogramming" effect is buffered out by p53.

There are several studies demonstrating Esrrb not only has the ability to replace the C-myc and Klf4 to induce pluripotent stem cells with Oct4 and Sox2, it also has the ability to induce Sox2 directly in a small population of differentiated cells, as well as Oct4 up-regulation activity [19, 70, 279]. These results strongly support that Esrrb is a central factor in the induction of pluripotency and obviously the question raised is: What function does the induction/activation of Esrrb play in cancer cells? Cancer cells that show stem cell features are associated with higher potential for metastasis and resistance to radiotherapy and chemotherapy, in addition, they account for the cancer recurrence [204, 211]. These cancer cells have rapid self-renewal and are associated with severe disease outcome. Esrrb overexpression led to the decrease in proliferation of DU145 cell xenograft tumor and this result eliminated the possibility that Esrrb dedifferentiate cancer cells [274]. We speculate that this tumorgraft-shrinking effect is due to the ability of Esrrb to activate p53 and p53 associated cell cycle arrest, proliferation inhibition, cell cycle arrest and apoptosis induction.

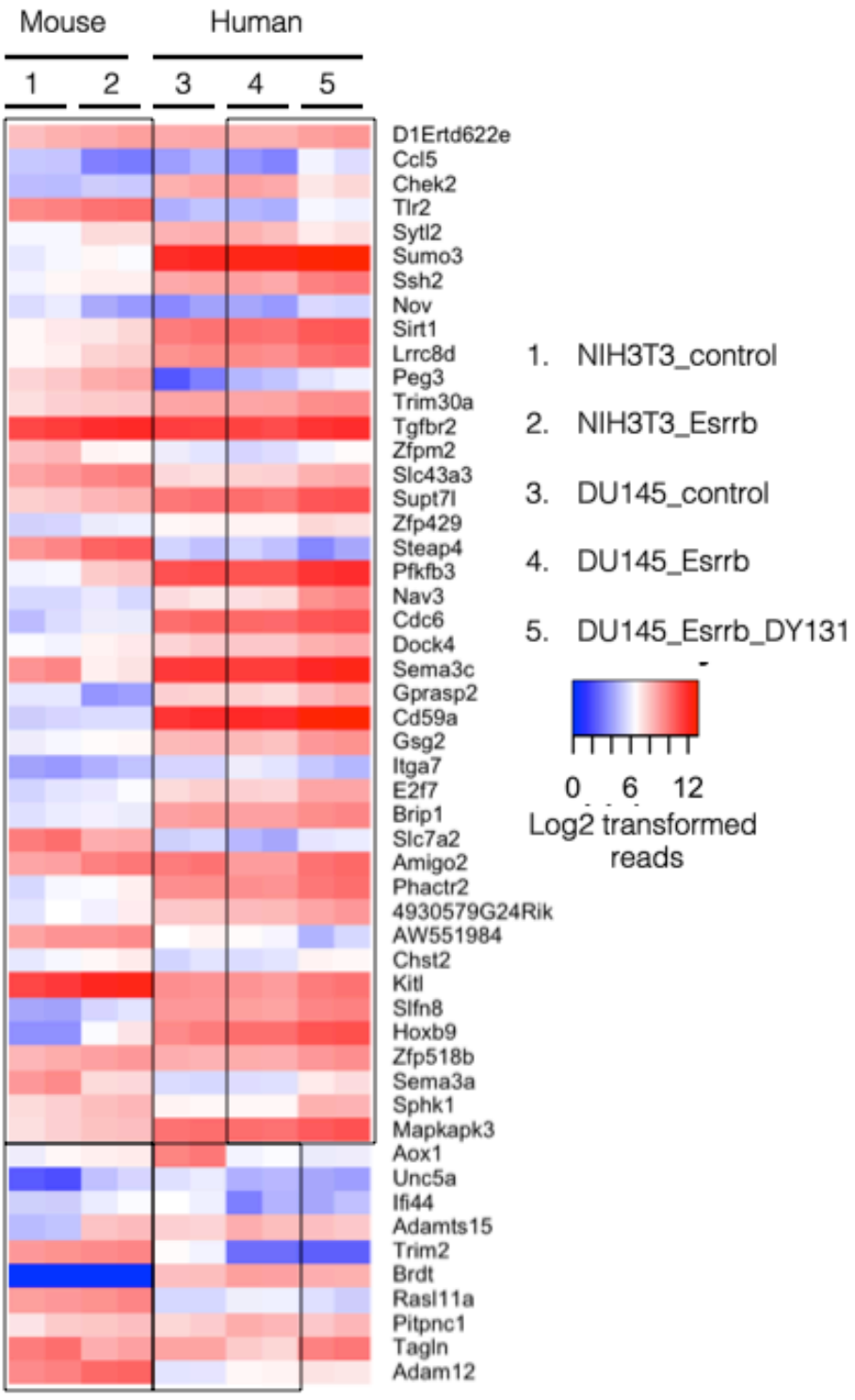


Figure V-1 Esrrb Responsive Genes in both human prostate cancer cells and mouse embryonic fibroblast cells.

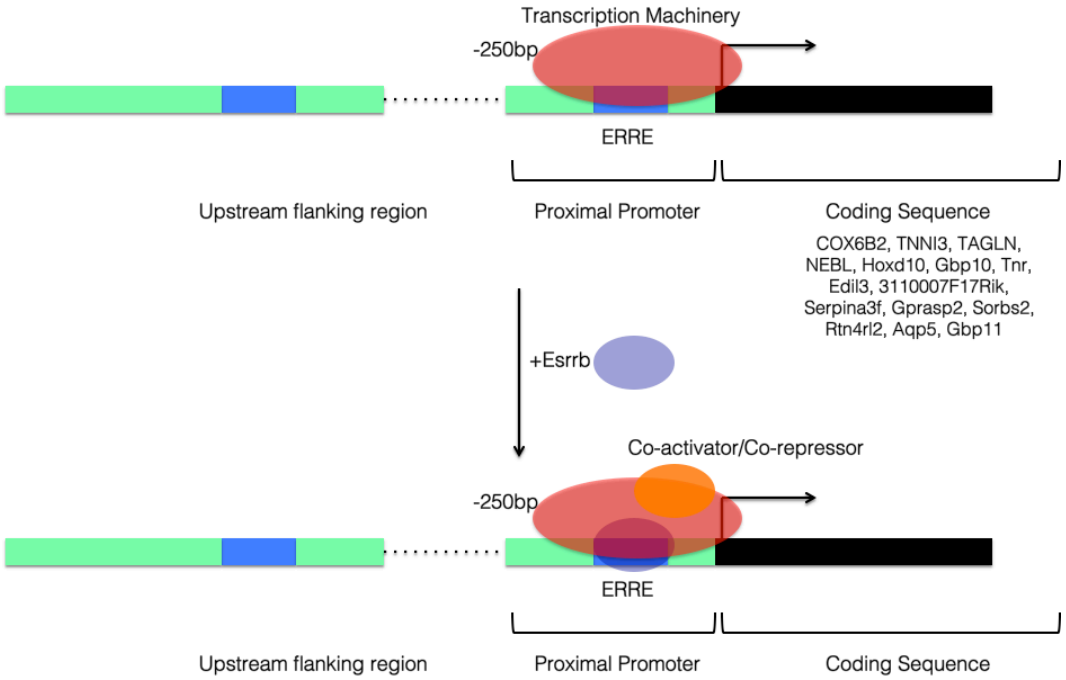


Figure V-2 Illustration of the mechanism of Esrrb regulation of its target genes.

Cox6b2, Tnni3, Tagln, Nebl, Hoxd10, Gbp10, Tnr, Edil3, 3110007F17Rik, Serpina3f, Gprasp2, Sorbs2, Rtn4rl2, Aqp5, Gbp11 are Esrrb regulated genes that have at least one copy of ERRE/SFRE in their proximal promoter region. When Esrrb is not expressed, the transcription of these genes is based on the basal transcription machinery. When Esrrb is expressed/activated, Esrrb binds to the ERRE in the promoter and recruit/dissociate co-activators or co-repressors to regulated the transcription.

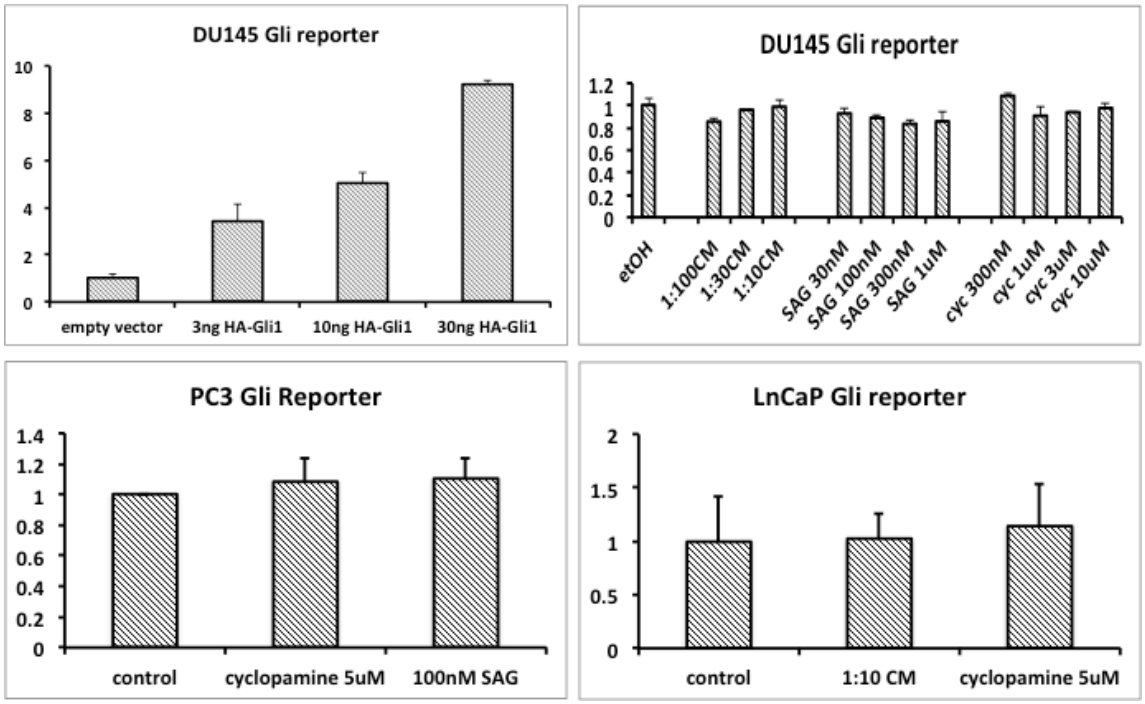


Figure V-3 Gli reporter assays performed in human prostate cancer cells DU145, PC3 and LnCaP do not response to Hh-signaling modulators.

Upper left: DU145 cells are co-transfected with 8XGliRE-Luciferase reporter, Renilla and different amount of HA-Gli1 expression vector. Gli1 transfection is used as control for reporter assay. Upper right and lower panel: 3 human prostate cancer cell lines, DU145, PC3 and LnCaP were transfected with 8XGliRE-Luciferase reporter and Renilla reporter. Transfected cells are further treated with Shh enriched CM, SMO agonist (SMO), or cyclopamine/cyc (5uM).

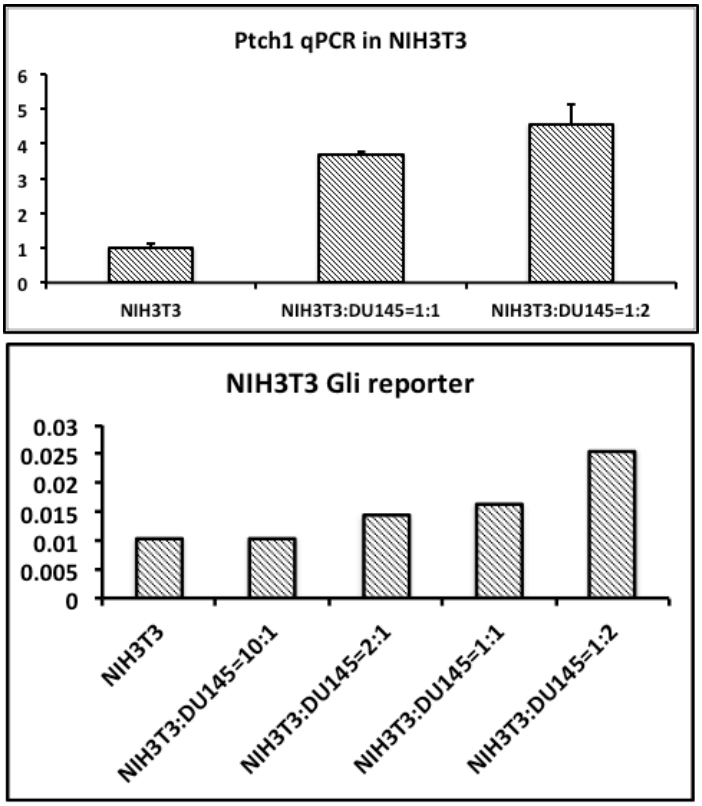


Figure V-4 Co-culture of NIH3T3 cells with prostate cancer DU145 cells

Upper panel: NIH3T3 and DU145 cells are mix cultured at different ratios (as indicated) for 48 hrs. Total RNA of the co-culture system were then isolated and purified and subjected to cDNA library preparation. qPCR was performed using mouse specific Ptch1 and GAPDH primers and gene expression value is calculated using standard curve method. Ptch1 expression value are firstly normalized to GAPDH expression value and the resulted number are further normalized to that of control condition (NIH3T3 alone). Lower Panel: GliRE-luciferase and control Renilla reporter transfected NIH3T3 cells are mix cultured with DU145 cells at different ratios (as indicated) for 48 hrs. Co-culture system was disrupted by applying detergent, and luciferase activity was measured using Dual-Luciferase system. Luciferase measurement normalized to Renilla measurement.

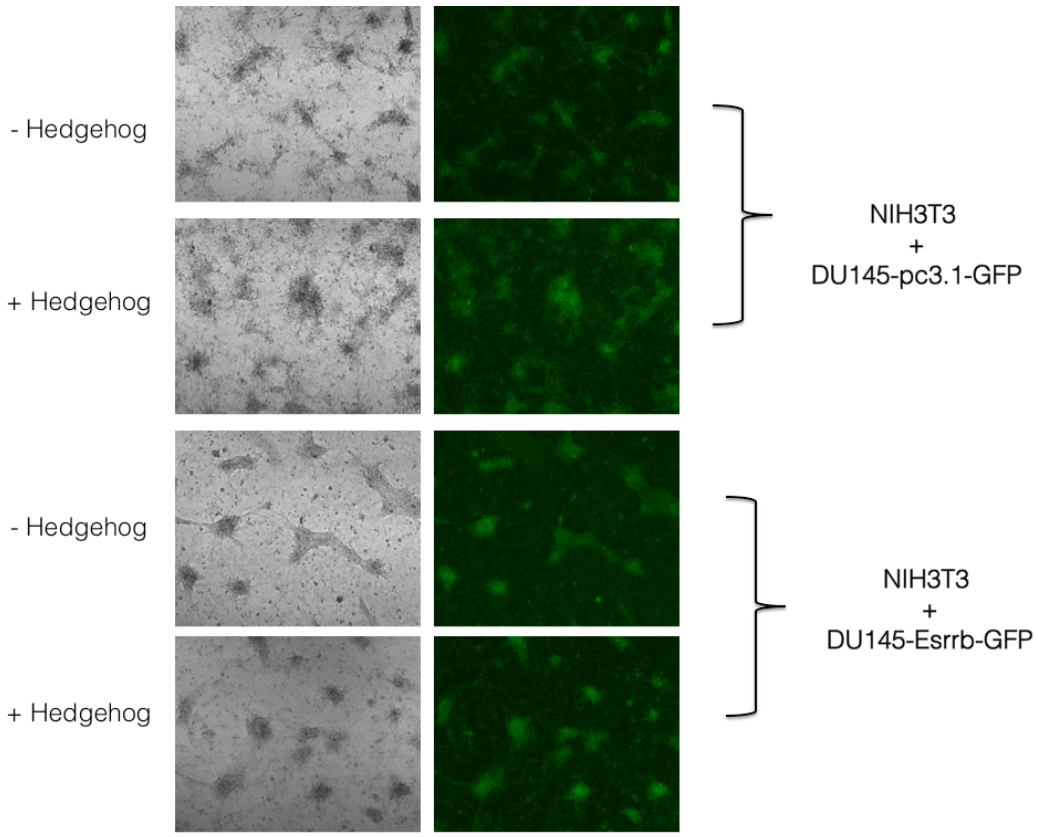


Figure V-5 Microscopic image of NIH3T3-DU145 co-culture system

DU145-pc3.1 and DU145-Esrrb cells were stably transfect with GFP, resulted model cells were seeded in 6-well-dish at 1:1 ratio for 24 hrs. Left panel: white light image showing all cells. Right panel: GFP labeled DU145-pc3.1 or DU145-Esrrb cells. Hh treatment in the upper two panels showed Hh treatment increased the cancer cell colony size. Esrrb change the morphology of DU145 colony.

APPENDIX I

Inhibition of Gli/hedgehog-signaling by the "cancer bush" Sutherlandia/ *Lessertia frutescens* extract in prostate cancer

Abstract

Sutherlandia frutescens is a medicinal plant from South Africa, and is traditionally used to treat various types of human diseases, including cancer. Our lab's studies show that many compounds are proposed to suppress prostate cancer by inhibiting the Hedgehog (Hh) signaling pathway. Here we hypothesized *Sutherlandia frutescens* can inhibit Hh-signaling in prostate cancer. *Sutherlandia frutescens* methanol extract can inhibit Hh-signaling revealed by decreased mRNA concentration of Gli1 and Ptch1 with the Hh-signaling stimulated. The Hh-signaling inhibitory effect from *Sutherlandia* extract is also confirmed by RNA-seq analysis. In addition to canonical Hh-responsive genes Gli1 and Ptch1, we distinguished new Hh responsive genes in TRAMPC2 mouse prostate cancer cell line. *Sutherlandia* extract is capable of inhibiting 50% of Hh-signaling induced genes. *Sutherlandia frutescens* extract induces genes with additional biological functions that are important for immune response and cell proliferation. In conclusion, *Sutherlandia frutescens* has a strong Hh-signaling inhibitory function that potentially leads to a second line Hh-signaling inhibitor.

Introduction:

Some of the results in this appendix are summarized in the manuscript "Inhibition of Gli/hedgehog signaling by the "cancer bush" Sutherlandia frutescens extract in prostate cancer" by Hui Lin, Glenn Jackson, **Yuan Lu**, Sara Drenkhahn, Kevin Fritsche, Valeri Mossine, Cynthia Besch-Williford, Korey Brownstein, Nicholas Starkey, William Folk, Yong Zhang and Dennis Lubahn (Re-submitted for publication).

"*Prostate cancer is the second leading cause of cancer-related deaths in men, behind only lung cancer. One in six men will suffer prostate cancer during his lifetime and one in thirty-six die of this disease [208]. The latest American Cancer Society report estimates 238,590 new cases of prostate cancer will be diagnosed in the United States in 2013 and more than 29,720 men will die of the disease in the same year. Prostate tumors can be treated by hormone antagonists, hormone ablation, and chemotherapy, however, many tumors recur and acquire resistance to the first line of treatment, making it difficult to efficiently control and cure the advanced-stage prostate cancer. In addition, these treatments have adverse side effects. Therefore, novel treatments that can specifically target abnormal signaling pathway(s) in prostate cancer but have fewer side effects are being sought [112, 169, 176].*

The Hedgehog (Hh) signaling pathway includes the ligands (Sonic hedgehog (Shh), Desert hedgehog (Dhh) and Indian hedgehog (Ihh)), the transmembrane proteins Patched (PTCH), and Smoothened (SMO) that can release the Gli zinc-finger transcription factors from the microtubules. During embryogenesis, the Hh pathway regulates cell proliferation, differentiation and patterning. Hh pathway activity decreases in most tissues during postnatal development. In adults, the Hh-signaling pathway is active in several tissues and is important for the maintenance of adult stem cell [90]. Deregulation of Hh-signaling during embryonic development causes physical malformations such as fused fingers, rib and facial abnormalities, whereas increased Hh-signaling in adults often leads to cancer, such as Medulloblastoma, basal cell carcinoma [169], small cell lung cancer [257], colorectal cancer [11], pancreatic

adenocarcinoma [235] and advanced prostate cancer [112]. Specific inhibitors against the Hh-signaling pathway shrinks the tumor in preclinical tumor xenograft models [51]. Several Hh-signaling inhibitors are currently in clinical trials and Vismodegib has recently been approved by the US Food and Drug Administration (FDA) [52, 169, 197, 249].

In the prostate, Hh-signaling is essential for the regeneration of the epithelium. Enhanced pathway activity transforms progenitor cells into tumorigenic cells and may promote the transition of localized cancer into metastatic prostate cancer [112]. Blocking the Hh-signaling using Cyclopamine or RNA interference of Gli expression suppresses the proliferation of several human prostate cancer cell lines [201]. In spite of its potent inhibition, Cyclopamine is not a favorable agent because of its rapid clearance, non-specific toxicity and instability. More importantly, it has off-target effects at higher concentrations [37, 169]. Therefore, screening for novel inhibitors against Hh pathway may be an effective way to inhibit prostate cancer proliferation and tumorigenesis.

Sutherlandia frutescens, commonly known as the "cancer bush" by South African healers, is a medicinal plant traditionally used to treat many human diseases, including cancer [29, 188, 244]. The ethanol extract of Sutherlandia frutescens showed dose-dependent anti-proliferative and apoptotic effects on several human tumor cell lines including the breast cancer cell line MCF-7. Similar inhibition can also be

achieved using an aqueous Sutherlandia frutescens extract [14, 226, 251]. Chinkwo et al. demonstrated that an aqueous extract induced cytotoxicity in cervical carcinoma and Chinese Hamster Ovary (CHO) cells [42]. Currently, Sutherlandia frutescens is used to treat symptoms of infection on HIV/AIDS patients, though it may interfere with the efficacy of anti-retroviral drugs [12, 108]. Nevertheless, the molecular mechanism and signaling pathway(s) that are perturbed by Sutherlandia frutescens have been largely unclear. Previous studies by in our lab demonstrated that several botanical compounds potentially prevent prostate cancer via inhibition of the Hh-signaling pathway [210]. Hui Lin in our lab also showed that Sutherlandia frutescens extract has the ability to inhibit Hh-driven Gli reporter activity as well as TRAMP prostate cancer incidence. In this study, we hypothesized that the anti-cancer effects of Sutherlandia frutescens were due to the inhibition of the Hh-signaling pathway. We observed significant inhibition of Hh-signaling by Sutherlandia frutescens extract.

Materials and Methods

Preparation of Sutherlandia frutescens methanol and ethanol extract:

Sixty tablets (300 mg) from one bottle of Phyto Nova Sutherlandia supplement (Thebe Natural Medicines (Pty) Ltd., Cape Town, South Africa) were ground into powder and then extracted with methanol. The mixture was sonicated and filtered through Whatman #4 filter paper and then centrifuged to separate the residual solids. The methanol was removed by rotary evaporation under a vacuum at 70 ˚C. The extract was then re-suspended in tissue-culture grade ethanol to yield a concentration of 50

mg/mL (dry weight of Sutherlandia frutescens per mL of ethanol, referred as Sutherlandia frutescens methanol extract (SLME)). Separately, 10 gram of grounded Sutherlandia leaf was added to 250 ml of 95% ethanol, mixed well by stirring several times and kept at room temperature overnight. The supernatant was harvested, and centrifuged at 2,000 g for 15 min and sterilized using 0.22μm filters. Before doing the experiment, the tubes with 1 ml of extract was dried down using speed-vacuum, and resuspended into 50 μl DMSO, making a final extract concentration of 84mg/ml.

Cell culture and reagents

Cell culture reagents were purchased from Life Technologies (Carlsbad, CA) unless otherwise indicated. All cell lines were obtained from the American Type Culture Collection (ATCC). Mouse prostate cancer cell line TRAMP-C2 was cultured in complete RPMI 1640 medium supplemented with 10% Fetal Bovine Serum (FBS), insulin, and DHT. Shh Light II cells (JHU-68) were derived from the NIH/3T3 cell line that was co-transfected with Gli-responsive firefly luciferase reporter and constitutive Renilla-luciferase reporter. These cells were maintained in DMEM medium supplemented with 10% newborn calf serum (NBCS), 0.4 mg/mL G418, 0.15 mg/mL zeocin, 4 mM L-glutamine, 0.05% HEPES and 1.5 mg/mL sodium pyruvate.

Conditioned medium (Hh-CM) was generated from an HEK293 cell line overexpressing the Shh-N-terminal peptide (HEK293-ShhN, a kind gift from Dr. Phillip Beachy, Standford University), which can bind to and stimulate the Hh pathway.

HEK293-ShhN cells were grown to 80-90% confluence in DMEM medium containing 10% FBS, 1% Pen/Strep and 40 mg/mL G418. The medium was replaced with DMEM containing 2% FBS, 1% Pen/Strep. After 24-30 h, the supernatant was collected and filtered through a 0.22 μm filter and stored at -80 ˚C until use."

Here begins my work not in the above manuscript.

Reverse Transcriptase PCR and Real time PCR:

Total RNA was isolated and purified from TRAMPC2 cells using RNeasy kit (Qiagen). 1000ng of total RNA was used to create cDNA libraries using Superscript III Reverse Transciptase (Invitrogen) with random primers and oligodT. cDNA concentration was determined using SYBR qPCR (iQ SUPERMIX, BioRad) on ABI7500 system. 95 degree, 30 seconds; 60 degree, 40 seconds; 72 degree, 40 seconds. Primer sequences are listed in supplement material.

Deep sequencing and Differentially Expressed Genes:

TRAMPC2 cells were treated with 8ug/ml or 80ug/ml Sutherlandia ethanol extract with or without CM for 24 hours. Total RNA was extracted and quantified. 2500ng total RNA from 2 biological replicates was used to generate cDNA libraries using TruSeq Stranded mRNA Sample Preparation kits (Illumina) according to manufacturer's manual. RNA and cDNA library quality was determined by University of Missouri

DNA core. The deep sequencing was performed by MU DNA core using Illumina HiSeq 2000. The sequencing reads were trimmed and filtered using FASTX-Toolkit, and mapped to genome using Bowtie and TopHat [122, 237]. Gene expression is determined by gene raw read counts by an in-house tool MULTICOM-MAP[221-223]. Differentially expressed genes were calculated by edgeR [194]. Differentially expressed genes are determined by Log2 Fold Change (FC) < -1 or Log2 FC>1, $p<0.05$, FDR<0.05.

Result

Sutherlandia altered gene expression

8ug/ml Sutherlandia ethanol extract did not result in any differentially expressed gene that met our requirement (Log2FC<-1 or Log2FC>1, $p<0.05$, FDR<0.05). However, 80ug/ml Sutherlandia ethanol extract altered the mRNA concentration of 151 genes, including 40 genes down-regulated, and 111 genes up-regulated (Figure Appendix-1a). We performed Gene Ontology analysis and KEGG pathway analysis on the differentially expressed genes. The up-regulation of IL6, IL15, IL18BP, CXCL9, CXCL10, CXCL13 and TLR3 showed the primary function of Sutherlandia is immune and inflammatory related (Figure Appendix-1b). Interestingly, KEGG pathway analysis returned two pathways that are potentially affected by Sutherlandia treatment. On one hand, IL6 and CXCL10 are transcriptional targets of NFκB, the up-regulation of these two genes indicates the activating of NFκB. On the other hand, cytokines IFNB1, IL6, IL15 along with cytokines receptor IL12RB1, IL15RA all showed at least 2-fold

up-regulation, indicating the over-activation of cytokine-cytokine receptor signaling and potentially downstram JAK-STAT signaling (Figure Appendix-1c, d).

Hedgehog-signaling altered gene expression

Hh ligand-enriched Conditioned Medium (CM) treatment to TRAMPC2 cells for 24 hours led to 90 differentially expressed genes, with 70 genes significantly up-regulated more than 2 fold ($log2FC>1$, $p<0.05$) and 20 genes significantly downregulated more than 2 fold ($log2FC<-1$, $p<0.05$) (Figure Appendix-2a). Comparing this set of differentially expressed genes to a new set of Hh-signaling responsive genes found in mouse embryonic fibroblast cell NIH3T3 from our lab, we found 19 overlapping genes including Mt2 and Stc1, whose transcripts are inhibited by Hh ligand treatment. These are Fzd4, Trp63, Scara5, Rbm20, Rasl11b, Pkdcc, Pare8, Igfbp5, Hes1, Grb14, Cyb14, Cyb561, Atp6v0a4, Angpt4, Ace as well as classical Hh-signaling target genes Gli1, Ptch1 and Hsd11b1 (Figure Appendix-2b). The presence of shared Hh responsive genes indicates these 19 genes are common Hh-signaling target genes independent of cell type. Other than these 19 common Hh-signaling target genes, we also found new Hh target genes that have never been reported before including Penk (proenkephalin) and Cacna2d2 (Voltage-dependent calcium channel subunit alpha2delta-2).

Sutherlandia represses Hedgehog-signaling pathway.

To find out whether Sutherlandia extract has a potential Hh-signaling pathway inhibitory effect, we co-treat 80ug/ml Sutherlandia extract with Hh-CM and performed

gene expression profiling. 35 of the 70 genes that are up-regulated by Hh-CM were repressed by Sutherlandia treatment by more than 2 fold, 7 of the 20 genes that are down-regulated by CM were stimulated by Sutherlandia treatment by more than 2 fold. The Hh-signaling altered genes that can be repressed by Sutherlandia include the classical Hh-signaling target genes Gli1 and Ptch1, as well as newly distinguished gene Hsd11b1 and Penk (Figure Appendix-3). Other than Hh inhibition activity, the differentially expressed genes also contain the genes that carry the immune and inflammatory, including Il6, Il15, Cxcl9, Cxcl10, Cxcl11, Cxcl12, Cxcl13, Tlr3, Tlr12, Ifnb1 and Il12rb1.

To confirm the Hh-signaling inhibitory effect, we measured Gli1, Ptch1 and Hsd11b1 transcripts concentration after Hh-CM, and Hh-CM plus Sutherlandia extract treatment in TRAMPC2 cells. Consistent with RNA-seq results, all 3 genes were stimulated by Hh-CM treatment, while the administration of Sutherlandia inhibited the stimulation (Figure Appendix-4 a, b, c).

Discussion

We have showed that Sutherlandia extracts have strong anti-hedgehog effects, as well as important function in regulating immune response-related genes, confirming Sutherlandia has potential health benefit effects in infection disease control and cancer prevention.

Sutherlandia, also known as cancer bush, has long history being used as anti-virus and anti-cancer herbal medicine in South Africa [162]. Aqueous Sutherlandia extract was shown to inhibit breast cancer cell growth [251]. We also found Sutherlandia extract has growth inhibition effect in several prostate cancer cell lines. In addition, we also found the administration of Sutherlandia extract to TRAMP (TRansgenic Adenocarcinoma of Mouse Prostate) mouse model can decrease the incidence of small size advance prostate cancer PDC (Poorly Differentiated Carcinoma), indicating Sutherlandia has strong cancer treatment as well as cancer prevention effect (Hui Lin, in revision).

Retrospectively, the first Hh-signaling pathway inhibitor was found in a botanical source even before the Hh-signaling pathway itself was discovered. Cyclops-like birth deformation, including phenotypes of foetal hypophysial aplasia, foetal thyroidal and adrenal hypoplasia, and foetal gonadal hypertrophy accompanied with midline differentiation abnormality of internal organ and hindbrain, of sheep offspring from mother that grazed on corn lily (*Veratrum californicum*) during 11 to 14 days of pregnancy was found due to the alkaloid Cyclopamine enriched in *Veratrum californicum* [242]. Cyclopamine was later found to be Hh-signaling inhibitor by binding to SMO [36]. With the hypothesis that the cancer cell proliferation inhibition of Sutherlandia extract is due to its potential Hh-signaling pathway inhibition, we firstly distinguished a group of Hh-signaling responsive genes in TRAMPC2 cells, and we further found Sutherlandia extract has the ability to repress 50% of the responsive genes by more than 2 fold, suggesting Sutherlandia extract has strong Hh-signaling inhibitory

effects. Interestingly, Cyclops foetal adrenal synthesizes much less cortisol, cortisone and corticosterone than the maternal adrenal, indicating corticosteroid biosynthesis is also strongly related to *Veratrum californicum* grazing [242]. From our Hh altered genes in TRAMPC2 cells as well as Hh-altered genes in embryonic fibroblast cells NIH3T3 distinguished by RNA-seq in Chapter III, we found the cortisol-cortisone conversion enzyme coding gene Hydroxysteroid Dehydrogenase 11 Beta 1(Hsd11b1) was strongly response to Hh ligand, while Sutherlandia extract treatment, or SMO inhibitor treatment can repress the Hh-signaling pathway's effect on this gene [273] (Figure Appendix-5a). We can conclude that Hsd11b1 is an Hh-signaling responsive gene and the decreased cortisol and cortisone concentrations in the adrenal of Cyclops fetus are a result from *Veratrum californicum* grazing of mother sheep by Cyclopamine inhibition of Hh-signaling pathway. What caught our interest is what is the function of Hsd11b1 in prostate cancer cells. Hsd11b1 is also present in human prostate cancer cells PC3 and LnCaP, and the Hsd11b1's 11-dehydrogenase activity is preserved while the 11-reductase activity is not [60, 166], indicating Hsd11b1 is important for maintain the concentration of glucocorticoid in prostate and prostate cancer cells. Our result strongly correlates prostate cortisol/ cortisone concentration to the Hh-signaling pathway, revealing cortisol/ cortisone concentration alteration maybe an important factor for prostate cancer development and that anti-Hh treatment can reverse these detrimental changes.

Another new Hh-signaling responsive gene we found is Proenkephalin (Penk). Penk was shown to be a hormone ligand for the Opioid Growth Factor Receptor (Ogfr)

and is a negative regulator of cell proliferation and tissue organization. When Penk is synthesized and binds to nuclear located Ogfr, an intracellular signaling pathway downstream of Ogfr is initiated and eventually causes the cell to enter G0 phase and stop its own mitosis. Penk is a prostate stroma marker and gene expression analysis showed that Penk concentration is lower in prostate cancer than in normal prostate [85]. It is surprising to find that Penk is expressed in TRAMPC2, while DU145 is completely Penk null from another gene expression profile we have, indicating TRAMPC2 cells have stromal features potentially from Epithelial Mesenchymal Transition (EMT). Even more surprisingly, we found Penk is Hh responsive gene and Sutherlandia extract treatment decreases either basal level or Hh-stimulated Penk transcript concentration. Although Hh-signaling activation is well known to influence cell proliferation and cell cycle progression, the fact that Hh-signaling activation led to Penk up-regulation does not contradict this role. In a paracrine Hh-signaling system involving epithelium/ tumor cells and stroma cells, the Hh-responsive stromal cells secret growth factors to facilitate the proliferation of epithelium/ tumors without rapidly growing and dividing, which could be explained by the expression of Penk in stromal cells. The Penk up-regulation in response to Hh keeps the stromal cells in G0 and secreted growth factors stimulating tumor cells proliferation.

Although Sutherlandia extract showed repression of a large portion of the Hh-response genes, we do not think that Sutherlandia extract contains SMO inhibitor(s) like cyclopamine, GDC0449 or DY131, otherwise it would repress all of the Hh responsive genes. We propose Sutherlandia extracts contain one or more active

components that alter the activity of downstream Hh-signaling pathway, which interact with other signaling pathways, or alternatively they act at the level of Gli transcription factor level in a promoter specific manner.

In addition to the discovery of Hh-signaling inhibition effect, we also found a set of 77 genes up-regulated by Sutherlandia treatment with or without Hh-signaling activation. GO analysis showed that the majority of these genes are immune response related, including Cfb, Cfh, Gbp2, Gbp4, Gbp5, Gbp10, Cxcl9, Cxcl10, Cxcl11, Cxcl13, IL6, IL18bp, Oasl1, Rsad2, Sp110, Tgtp1, Tgtp2, Tlr3, Tnfsf10 and Vnn1. While most of the up-regulated genes are shown to be Hh-signaling independent, revealed by the fold change similarity with or without Hh treatment, we do find 5 genes, Agap2, Aw112010, Gda, Cxcl11 and Npsr1, which are stimulated much more in response to Sutherlandia when Hh-signaling is activated, indicating that Hh-signaling facilitates these genes' response to Sutherlandia.

In summary, our gene expression survey of Hh-signaling responsive genes and Sutherlandia responsive genes in mouse prostate cancer TRAMPC2 cells showed Sutherlandia extract has strong Hh-signaling inhibition effects as judged by the number and percentage of Hh responsive genes that can be repressed by Sutherlandia treatment. Our RNA-seq results show that Sutherlandia is a strong anti-cancer, anti-Hh drug candidate with strong immune system boosting activities.

a

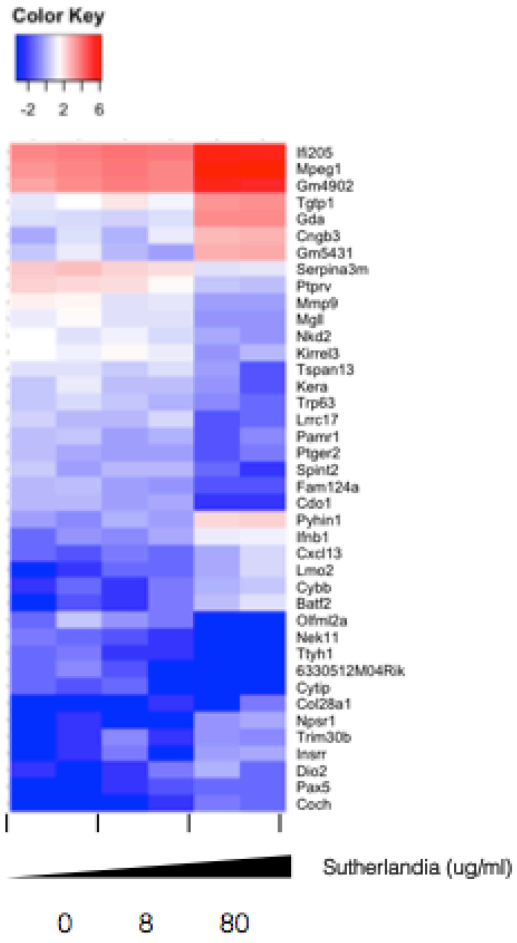


Figure Appendix-1. Sutherlandia alter genes in TRAMPC2 cells

(a) Gene expression values are represented by Log2 transformed normalized RNA-seq reads (Log2 count-per-million-reads).The gene expression values of 2 biological replicates of TRAMPC2 in different conditions(left panel: control, middle panel: 8ug/ml Sutherlandia, right panel: 80ug/ml Sutherlandia, are shown. Genes shown in here are top 20 genes that are up-regulated or down-regulated.

b

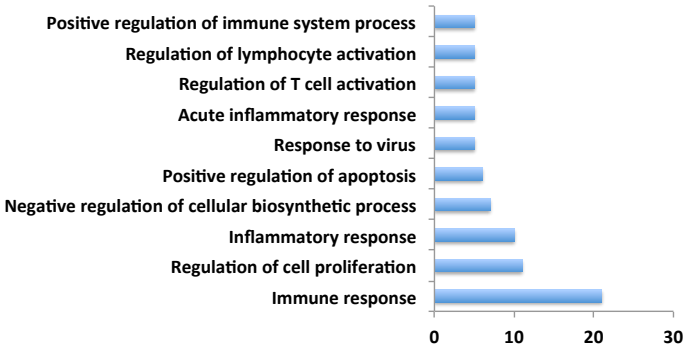
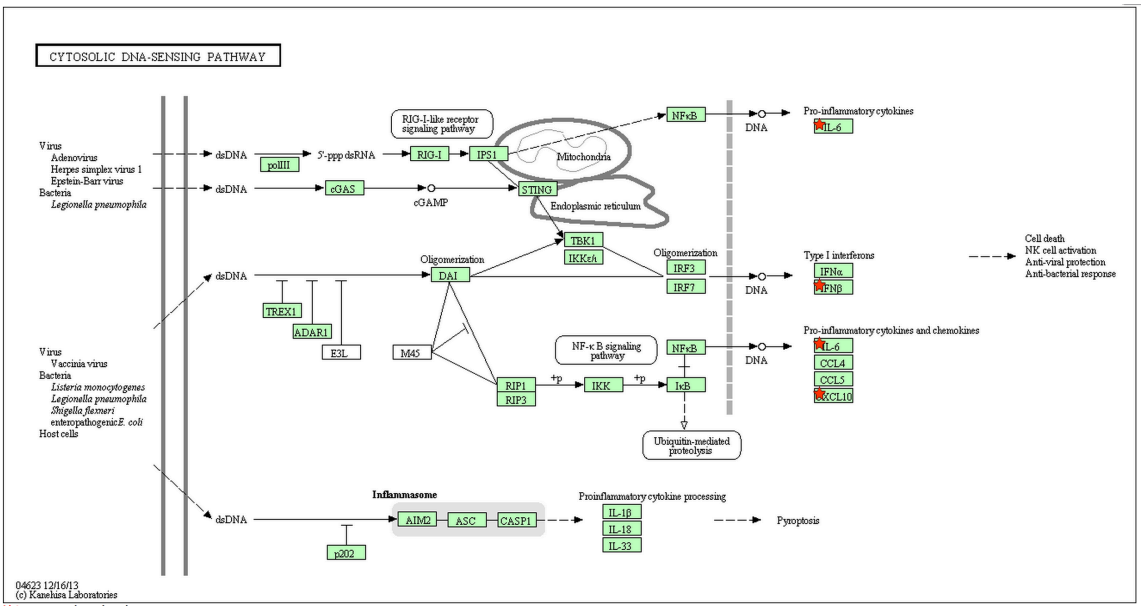


Figure Appendix-1 (Continued). Sutherlandia alter genes in TRAMPC2 cells

(b) Gene Ontology analysis of Sutherlandia responsive genes.

c



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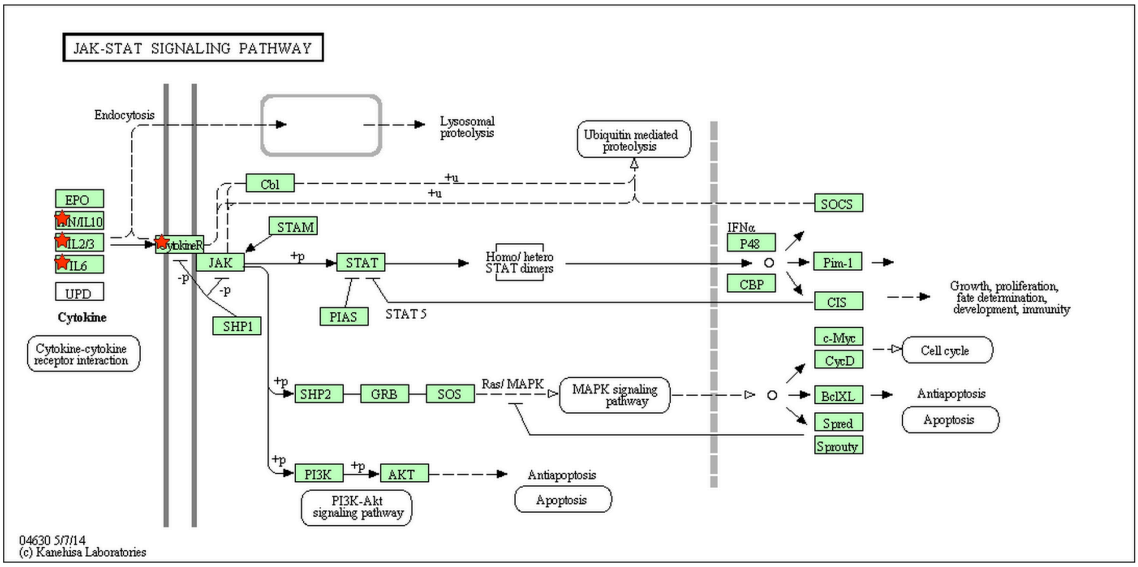


Figure Appendix-1 (Continued). Sutherlandia alter genes in TRAMPC2 cells

(c, d) KEGG pathway analysis of Sutherlandia responsive genes.

a

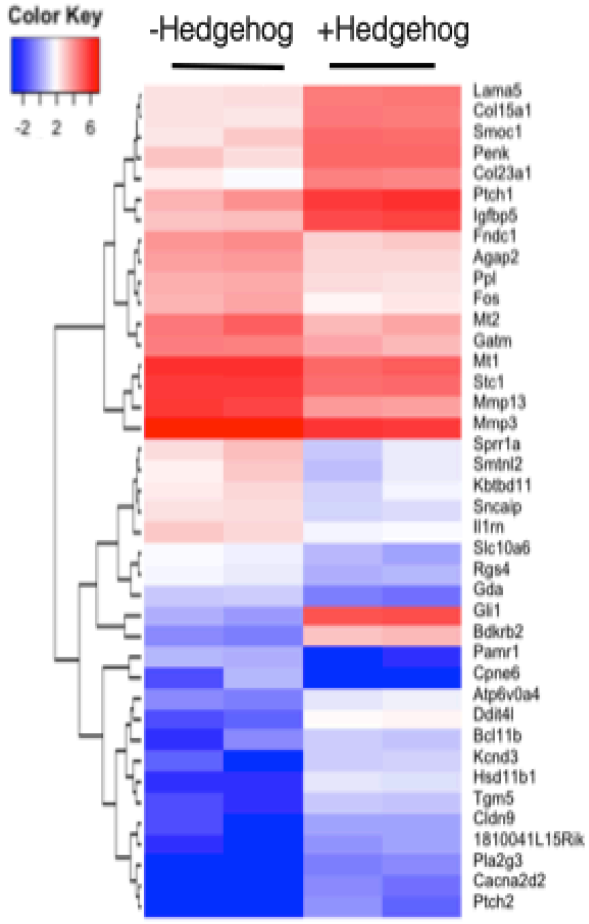


Figure Appendix-2. Heat map of differentially expressed genes in response to Hedgehog ligand for 24 hours

(a)Gene expression values are represented by Log2 transformed normalized RNA-seq reads (Log2 count-per-million-reads). The gene expression values of 2 biological replicates of TRAMPC2 in different conditions (left panel: no Hedgehog ligand, right panel: with Hedgehog ligand), are shown. Hedgehog ligand treatment led to 90 differentially expressed genes, genes shown in here are top 20 genes that are up-regulated or down-regulated.

b

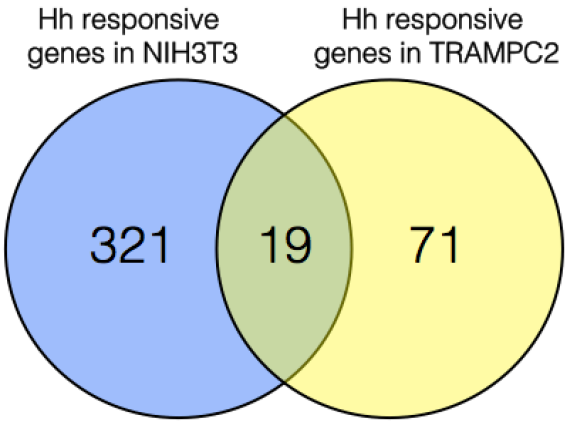


Figure Appendix-2 (Continued). Heat map of differentially expressed genes in response to Hedgehog ligand for 24 hours

(b) Venn Diagram showing the common the number of common Hh-responsive genes in NIH3T3 and TRAMPC2 cells

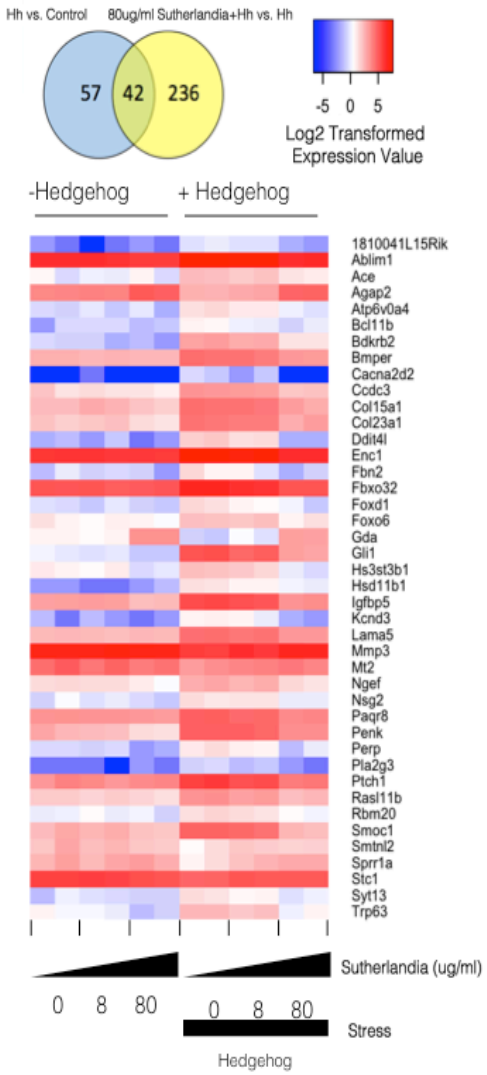


Figure Appendix-3. Heat map of Sutherlandia altered Hedgehog-signaling pathway target genes expression

Gene expression values are represented by Log2 transformed normalized RNA-seq reads (Log2 count-per-million-reads).The gene expression values of 2 biological replicates of TRAMPC2 in different conditions(left panel: no Hedgehog ligand, right panel: with Hedgehog ligand), each with different dose of Sutherlandia extract are shown. All genes are Hedgehog ligand responsive (Fold change>1 or <-1), while Sutherlandia extract has repressive effect on the Hedgehog ligand effect.

a b c

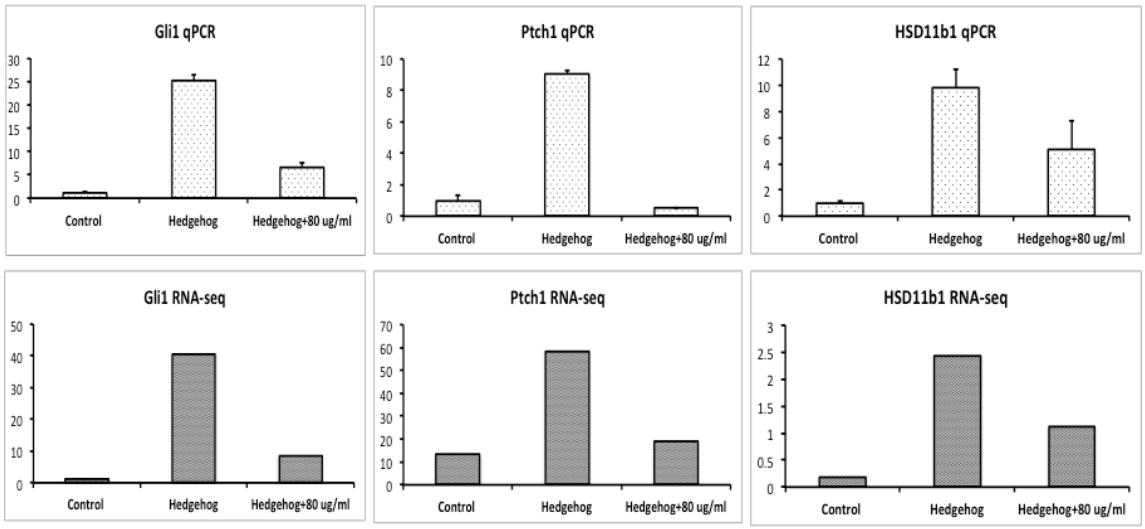
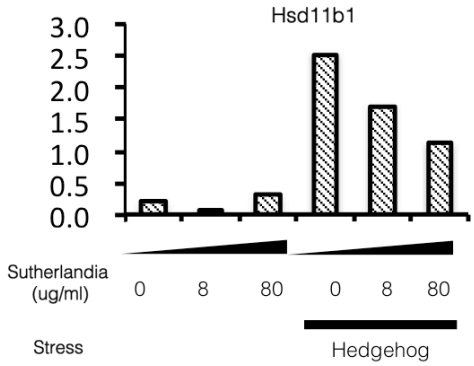


Figure Appendix-4. qPCR validation of RNA-seq result.

Quantitative PCR for (a) Gli1, (b) Ptch1, and (c) Hsd11b1 was performed on Hh ligand treated and Hedgehog ligand and Sutherlandia co-treated TRAMPC2 cells. Transcripts concentrations were normalized to control. In the lower figure, transcripts concentrations of (a) Gli1, (b) Ptch1, and (c) Hsd11b1 are represented by quantified sequencing reads, in the form of counts-per-million-reads.

a



b

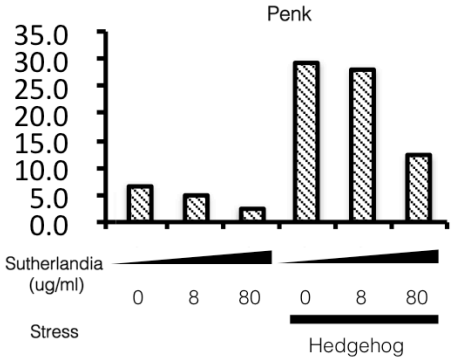
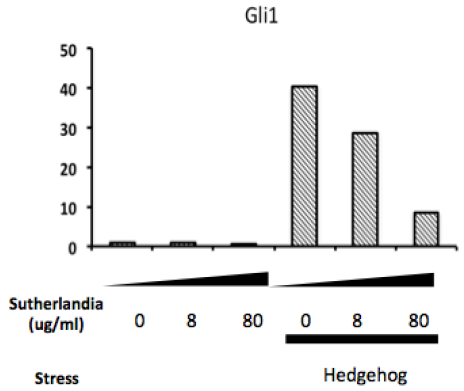


Figure Appendix-5. RNA-seq reads of Hsd11b1 and Penk

Transcripts concentrations of (a) Hsd11b1 and (b) Penk are represented by normalized deep sequencing reads, in the form of counts-per-million-reads (cpm). The cpm values are shown for different length of time and in the conditions with or without Hedgehog ligand.

c



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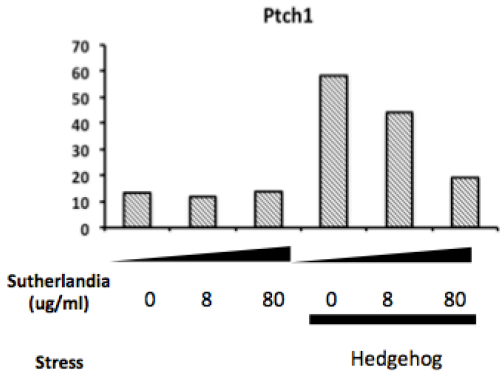


Figure Appendix-5 (Continued). RNA-seq reads of Hsd11b1 and Penk

Transcripts concentrations of (c) Gli1 and (d) Ptch1 are represented by normalized deep sequencing reads, in the form of cpm.

APPENDIX II

Using Next Generation Sequencing to Distinguish Fused Transcripts in Prostate Cancer Cells

Introduction:

Coding gene fusion is refer to two independent mRNA transcripts are fused together through chromosome translocation. When two partner genes A and B form gene fusion, part of gene A and part of gene B are linked together and finally form a chimeric protein product carrying the function from both gene A and B (Figure Appendix II-1). TMPRSS2-ERG gene fusion is a classical example of oncogenic gene-fusion in prostate cancer patient [140]. The transcription and translation from the genome alteration renders the overexpression of oncogene ERG and allows it to initiate its transcription regulatory function.

As the RNA-seq technic become an affordable and common tool nowadays, transcriptome-wide gene fusion discovery is technically possible. Many bioinformatics tools are also available for this task. For example, Tophat 2, deFuse, SOAPfusion and Fusionmap are all capable of calling gene fusion [81, 116, 146, 262]. While most of the tools require pair-end reads due to the nature of the algorithm (if the left read and right read of one fragment come from two different genes, then this fragment covers the fusion point of the fused transcript), Bowtie1-Tophat2 allows single-end reads to be the input for gene fusion discovery (if the left side of one read can be mapped to one exon of gene A, and the right side of the same read are mapped to the exon of another gene B, then this read is a supporting read for gene fusion A-B).

Due to the nature of our dataset from DU145 cells (single-end 50 base pair read), we applied Tophat2 to discover the potential gene fusions.

Material and Method:

DU145 cells were cultured in phenol red free RPMI1640 with 10% charcoal-stripped Fetal Bovine Serum (FBS). Total RNA was extracted and purified by RNeasy kit. 2500ng total RNA was used to generate cDNA libraries using TruSeq Stranded mRNA Sample Preparation kits (Illumina, San Diego, CA) according to the manufacturer's manual. RNA quality and fragment sizing of cDNA library were determined by the University of Missouri DNA core. Deep sequencing was performed by the MU DNA core using Illumina HiSeq 2000 following the manufacture's instruction. Around 18 million reads were generated in .fastq format.

Raw data are downloaded to user data directory on Lewis server.

To remove sequencing adaptor, FASTX-Toolkit (V 0.0.13) (hannonlab.cshl.edu/fastx_toolkit) is used:

```
-o directory/trimmed_1.fastq.%J
fastx_clipper
-a AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC
-i directory/1_ATCACG_L002_R1_001.fastq -Q 33
```

To trim off the low quality nucleotide at the end of reads, "qualtiytrim" command is used:

```
-o directory/trimmed_1_1.fastq.%J
fastq_quality_trimmer -t 20 -l 20
-i directory/trimmed_1.fastq -Q 33
```

After the trim, if read quality is still low judging on the quality score associated with each nucleotide, the low-quality reads are removed:

```
-o directory/trimmed_1_1_1.fastq.%J
fastq_quality_filter -p 90 -q 20
-i directory/trimmed_1_1.fastq -Q 33
```

Short reads were mapped to UCSC hg18 genome. Bowtie1 carried out regular read mapping. Reads that covered the exon-exon junction, as well as the reads that come from gene fusion product, were mapped by Tophat2 [122, 237].

```
-n 4
-R "span[hosts=1]"
tophat2 -p 4 --fusion-search –bowtie1 hg18 trimmed_1_1_1.fastq
```

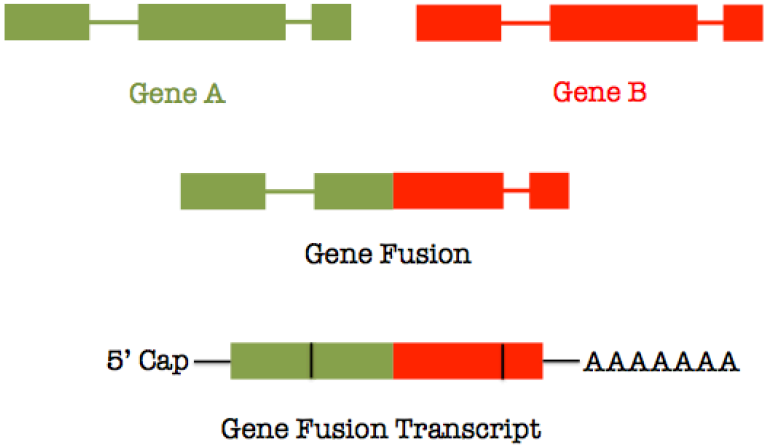
The resulted potential fusion candidates are further filtered through tophat-fusion-post:

```
-o tophat_fusion_post_test.o%J
-n 4
-R "span[hosts=1]"
tophat-fusion-post -p 4 --num-fusion-reads 1 --num-fusion-pairs 0 hg18
```

Results:

We found 424 potential gene-fusion candidates that have at least one read covering the fusion point (Table Appendix II-1). These candidate gene fusions have strong in silico evidence to be true gene fusion, but further bench work is required to prove their existence. If they are true gene fusions, clinical sequencing can be done to characterize the fusions' clinical relevance and further mechanism study can be carried on.

a



b

```
CCAAGGGCGTGAAGAGGAAAACGTTATTCAGGGAGAACGGCTCCAAGCCTAG
    GGGCGTGAAGAGGAAAACGTTATTCAGGGAGAACGGCTCCAAGCCAAGGGCCATCGTGGTGGATCCTGTTCATGG AATGGCTCCCGCAAAGAAGGGTGGC
    GGGCGTGAAGAGGAAAACGTTATTCAGGGAGAACGGCTCCAAGCCAAGGGCCATCGTGGTGGATCCTGTTCATGG AATGGCTCCCGCAAAGAAGAGTGGC
    GGGCGTGAAGAGGAAAACGTTATTCAGGGAGAACGGCTCCAAGCCAAGGGCCATCGTGGTGGATCCTGTTCATGG AATGGCTCCCGCAAAGAAGGGTGGC
     GGCGTGAAGAGGAAAACGTTATTCAGGGAGAACGGCTCCAAGCCAAGGGCCATCGTGGTGGATCCTGTTCATGG AATGGCTCCCGCAAAGAAGGGTGGCT
              GGAAAACGTTATTCAGGGAGAACGGCTCCAAGCCAAGGGCCATCGTGGTGGATCCTGTTCATGG AATGGCTCCCGCAAAGAAGGGTGGCAAGAATAAAAG
              GGAAAACGTTATTCAGGGAGAACGGCTCCAAGCCAAGGGCCATCGTGGTGGATCCTGTTCATGG AATGGCTCCCGCAAAGAAGGGTGGCAAGAATAAAAG
              GGAAAACGTTATTCAGGGAGAACGGCTCCAAGCCAAGGGCCATCGTGGTGGATCCTGTTCATGG AATGGCTCCCGCAAAGAAGGGTGGCAAGAATAACAG
              GGAAAACGTTATTCAGGGAGAACGGCTCCAAGCCAAGGGCCATCGTGGTGGATCCTGTTCATGG AATGGCTCCCGCAAAGAAGGGTGGCAAGAATAAAAG
              GGAAAACGTTATTCAGGGAGAACGGCTCCAAGCCAAGGGCCATCGTGGTGGATCCTGTTCATGG AATGGCTCCCGCAAAGAAGGGTGGCAAGAATAAAAG
                 AAACGTTATTCAGGGAGAACGGCTCCAAGCCAAGGGCCATCGTGGTGGATCCTGTTCATGG AATGGCTCCCGCAAAGAAGGGTGGCAAGAATAAAAGGGC
                 AAACGTTATTCAGGGAGAACGGCTCCAAGCCAAGGGCCATCGTGGTGGATCCTGTTCATGG AATGGCTCCCGCAAAGAAGGGTGGCAAGAATAAAAGGGC
                  AACGTTATTCAGGGAGAACGGCTCCAAGCCAAGGGCCATCGTGGTGGATCCTGTTCATGG AATGGCTCCCGCAAAGAAGGGTGGCAAGAATAAAAGGGCC
                    ACGTTATTCAGGGAGAACGGCTCCAAGCCAAGGGCCATCGTGGTGGATCCTGTTCATGG AATGGCTCCCGCAAAGAAGGGTGGCAAGAATAAAAGGGCCG
                                                          GGTGGATCCTGTTCATGG AATGGCTCCCGCAAAGAAGGGTGGCAAGAATAAAAGGGCCGTTCTGCCATCAACGAGGTGGTGACCCGAG
```

Figure Appendix II-1 Gene Fusion Model

(a) A model of gene fusion from chromosome relocation (b) An example of discovered gene fusion in DU145 cells. SMARCA4 (Chromosome 19) and SLAMF9 (Chromosome 1) are fused together, the contigs surrounding the fusion point are showed, and the gap is the fusion point

Table Appendix II-2 Gene Fusion Discovery Result (CD/online data set)

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