CHAPTER 4

Functional studies of SRD5A2
Introduction

Two 5 alpha reductase isozymes, designated types 1 and 2, have been identified by cDNA cloning in the human and rat [75]. The isozymes are approximately 250 amino acids in length, share 50% sequence identity, and are both integral membrane proteins of the endoplasmic reticulum. Despite these similarities, the isozymes have quite different kinetic, biochemical, and pharmacological properties. The type 1 isozyme has a low affinity for steroid substrates and a basic pH optimum and is sensitive to certain benzoquinolinone inhibitors [73, 83, 127]. The type 2 isozyme has a higher affinity for steroid substrates, an acidic pH optimum, and a unique sensitivity profile to certain 4-azasteroid inhibitors [17, 83].

The physiological role of the SRD5A2 protein is better understood due to the occurrence of natural mutations in the gene (SRDSA2) encoding the type 2 isozyme [36]. This isozyme is responsible for the embryonic synthesis of dihydrotestosterone that is required for the development of the external genitalia and prostate. Males homozygous for mutations in the type 2 gene fail to develop these reproductive structures [128]. To date, 20 different mutations in the SRDSA2 gene have been characterized at the molecular level in individuals with 5 alpha reductase deficiency [17, 18]. Three of these mutations (G34R, G196S, and R246W) affected the ability of the type 2 isozyme to interact with substrate and cofactor [18]. Despite the central role played by Sa-reductases in steroid hormone metabolism, little is known about functional domains in these enzymes. Although they share sequence identity with several viral proteins, they do not share obvious homologies with other NADPH utilizing proteins or enzymes that act on steroid substrates [75]. Here, we took two approaches to gain insight into structure-function relationships in SRD5A2. In the first, we screened for additional mutations in the SRD5A2 gene in subjects with 5 alpha reductase 2 deficiency. In the second, the biochemical consequences of 22 of these naturally occurring mutations for enzyme activity and stability were determined. In past SRD5A2 gene was only studied for mutation and polymorphism as discussed. In our studies, we want to know that exact role of SRD5A2 in cellular level and its role in apoptosis.
RESULTS AND DISCUSSION

SRD5A2 cloned in PBS with FLAG tag

SRD5A2 cDNA was subcloned in PBS from PCR 4 vector after attachment of flag tag sequence using PCR. Complete detail of the clone as shown in the Fig 51. The PCR products amplified using different combination of primer sets are shown in the Fig 51.

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**Figure 51:** Schematic diagram showing flag tag with SRD5A2 cDNA. Diagram showing position of EXON (E1 to E5), of SRD5A2 cDNA, represented in blue thick boxes, red thick box shows the position of flag tag attached in SRD5A2 cDNA. Combination of different primer groups result in different PCR product are shown (F4 & R4 = 854bp, Fsh & R4 = 839 bp, Fsh & R2 = 774bp and F2 & R2 = 315bp).

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**Figure 52:** SRD5A2 cDNA was subcloned with flag tag in pBS cloning vector. Gel showing the PCR product with the different primer set used to screening for the cloned product. 1; 100 bp ladder, 2; Flag+Fsh+R2(774 bp), 3; Flag+Fsh+R2 (839 bp), 4; F4 & R4 (854 bp), 5; F2 & R2 (315 bp), 6; 1kb ladder.
Construction of antisense plasmid for inhibition of SRD5A2

Full length cDNA of SRD5A2 was PCR amplified as described in Material and Methods section. The PCR product was cloned in pcDA3.1 mammalian expression vector in antisense direction.

Confirmation of antisense orientation of SRD5A2 cDNA

Orientation of SRD5A2 cDNA in pcDNA3.1 was confirmed by PCR using different set of primer as shown in the Fig 53 A. PCR product of the cDNA clone was run on the 2 % agarose gel and photographed as shown in the Fig 53 B.

Figure 53: Confirmation of antisense orientation of SRD5A2 cDNA in pcDNA 3.1 mammalian expression vector. PCR amplified SRD5A2 cDNA was run on a 2% agarose gel. SRD5A2 cDNA was subcloned into pcDNA 3.1 mammalian expression vector. A; The orientation of the cDNA was confirmed by PCR using appropriate primer set as described earlier. F2 and R2 primer used for SRD5A2 amplification was described in material and methods section. T7 is primer used from the pcDNA3.1 vector to confirm the direction of insertion. B; gel representing the PCR product of antisense clone. Lane 1; 1 kb ladder, lane 2; T7 & R2 (555 bp), lane 3; T7 & F2 (870 bp) and lane 4; F2 & R2.

Confirmation of SRD5A2 sequence identity in the antisense clone.

SRD5A2 cDNA clone was further confirmed for the orientation using sequencing as shown in the Fig 54.
Figure 54: Dendogram of sequencing SRD5A2 antisense clone.

Orientation of the SRD5A2 cDNA were confirmed by DNA sequence analysis & aligned using BLAST

SRD5A2 cDNA sequence was aligned with anti sense clone sequence using pair wise alignment. The antisence clone is perfectly in opposite direction in comparison of cDNA sequence of SRD5A2 gene sequence as shown in the

Figure 55: DNA Sequence of the antisense SRD5A2 clone was aligned in the opposite direction using BLAST. The nucleotide antisense SRD5A2 cDNA subcloned into pcDNA3.1 was aligned to the full length cDNA sequence of SRD5A2 from NCBI using BLAST. In the above figure it is clearly shown that sequence labeled as ‘Query’ is from antisence clone that from 71- 610 bp and the sequence labeled as ‘Sbjct’ is the SRD5A2 database result showing sequence from 881 to 344 bp.

Establishment of a stable cell line expressing antisense SRD5A2 RNA

Cell lines were co-transfected with pdEGFP and SRD5A2 antisense plasmids in 1:3 ratio using lipofectamine as mentioned in materials and methods section. G418 resistant clones were obtained after several weeks and were assessed for green fluorescence as shown in the Fig 51. Two of stable cell clones were selected and their expression of SRD5A2 mRNA was examined by standard RT/PCR method using SRD5A2 specific primers. These clones were named as AS-SRD-DU145a, AS-SRD-DU145b for DU145 cell lines, and AS-SRD-PC3a, AS-SRD-PC3b for the PC3 cell lines as shown in the Fig 51. Similarly both the cell lines were transfected with pcDNA 3.1
vector alone and taken as a vector control they were labeled as V-DU145 and V-DU145b for DU145 cell line and V-PC3a and V-PC3b for PC3 cell lines.

**Figure 56: Stably transfected DU145 and PC3 cell clones expressing antisense EGFP.** The DU145 and PC3 cell lines were co-transfected with the pdEGFP and SRD5A2 antisense gene construct were examined under Nikon fluorescence microscope. A; DU145 cell line B; PC3 cell line showing green GFP fluorescence, photographs were taken under 10 X and 40 X objectives.

**Characterization of stably transfected antisense clone**

To confirm the inhibition of expression of SRD5A2 using anti-sense RNA, we checked the SRD5A2 RNA level using Reverse Transcriptase PCR. Expression level was quantified by the Image J software. We observed a reduction of SRD5A2 mRNA level in antisense clones that is 44% and 42% inhibition in DU145 and PC3 respectively. Suggesting that there was stable reduction of SRD5A2 in stable cell clones. Cells transfected with only empty vector (pcDNA3.1) were used as controls showed no difference in the expression level of 18S rRNA as shown in the Fig 52.
Figure 57: Expression of SRD5A2 mRNA in the stably transfected antisense clones examined by RT-PCR. Upper panel lane 1: 100 bp DNA ladder, lane 2: 18S rRNA amplified using vector control, lane 3: 18S rRNA amplified using antisense clone, lane 4: SRD5A2 amplified using vector control, lane 5: SRD5A2 amplified from antisense clone. A: DU145 cells, B: PC3 cells. Lower panel showed the quantification of the gel showing above; A: DU145 cells, B: PC3 cells.

Growth analysis of anti-SRD5A2 cell clones.

Proliferation rate of the parental control and stable transfected antisense clones were examined by standard MTT assay. Only vector transfected clones were also used as negative control. Cell growth was monitored for 24, 48 & 72 h as shown in the Fig 53. Anti-sense SRD5A2 cell clones grew faster as compared to vector control cells. The statistical significance of the of the cell growth was determined by unpaired student t test having p value ≤ p 0.05.
Figure 58: Growth assays of SRD5A2 antisense clones. The bar diagram shows cell viability of DU145a and PC3b clones.
Downregulation of SRD5A2 mRNA expression affected cell invasion.

During metastasis, invasion of the basement membrane by tumor cells is a critical event. To study whether the inhibition of SRD5A2 is associated with metastasis of prostate cancer cells, the effect of SRD5A2 inhibition on cellular motility and invasive activity was evaluated. SRD5A2 is a microsomal protein and its role in cancer cell invasion is yet to be seen. SRD5A2 and vector transfected cells (1x 10^5) were plated in the upper chamber of transwell plate and after 48h number of cells migrated to the lower chamber were examined. Our results showed that SRD5A2 inhibition induced an increase in the cell invasion capability in comparison with vector controls. DU145 invasion rate was more in comparison with the PC3 cells as shown in the Fig 55.
Figure 60: Effect of SRD5A2 inhibition on cell invasion. Stable antisense clones were seeded on an upper filter well (8-μm pore) of transwell chambers. After 24 hours of incubation, cells in the upper side of the filter were removed and the invaded or migrated cells were fixed, stained, and counted. A: Photograph showing the DU145 and PC3 cell invade to the lower side of the chamber showing increased number of cell invaded in the antisense clone as in compression with vector control transfected cells. B: The bar graphs showing the cell count data representing percent cell invasion after the 48 h; bars, SD.

SRD5A2 expression and cell migration.

Cell migration has a central process in the development and maintenance of multicellular organisms. Tissue formation during embryonic development, wound healing and immune responses all require the orchestrated movement of cells in a particular direction to a specific location. Errors during this process have serious consequences, including, vascular disease, tumor formation and metastasis. An understanding of the mechanism by which cells migrate may lead to the development of novel therapeutic strategies for controlling, for example, invasive tumor cells. These cells showed enhanced cell migration as compared to the control cells. The percent cell movement was calculated by the standalone software T-Scratch provided by MATLAB. It was used to calculate the open area in the image first the software use the photographed of cell migration and make them in grayscale mode which contained on black and white color. The software make all the portion of the image having cell as black and rest area as white and the total white area that is called as open area was calculated. The open image area gives the percentage that is indirectly proportional to the percentage of
migration. The effects of antisense on migration of DU145 & PC3 cells. Antisense clones increase the migration of the DU145 and PC3 cells, the observed effects on cell migration was significant and effect cell proliferation growth of the cell lines. The cell migration was calculated using T-Scratch and showed that the stable antisence clone of DU145 & PC3 showed 50 % and 70 % cell migration respectively after the 92 h of study as shown in the Fig 56. Antisense clones of DU145 and PC 3 cells affect the cell migration as well the cell number and induce apoptosis. These observations support the results of previous studies of cell invasion studies suggesting that must some signaling pathways activated which may be responsible for the effects that we observed.
Figure 61: SRD5A2 antisence stable clone is for DU145 and PC3 cell lines were analyzed for the cell migration. A; Photograph showed the DU145 and PC3 stable transfected with vector control and antisence SRD5A2 gene. antisence cells showed increased motility. Thin arrow indicates the more open area and thick arrow showed the less open area. B; The percentage of migration were calculated using TScratch software. The bar diagram represents the percentage of the open area in the image. These percentages of open area represent the cell migration rate.
Discussion

Biological function of SRD5A2 in prostate cancer cells and its loss or gain of expression during prostate cancer progression strongly suggests that SRD5A2 appears to play a major role in prostate cancer. These findings, along with localization of SRD5A2 to chromosome 2 and demonstration of increased frequency of LOH and homozygous deletion near the SRD5A2 locus, further supports our hypothesis that SRD5A2 may function as a candidate tumor suppressor gene. For example, we find that transfection of the human SRD5A2 gene in sense and anti sense direction in two human prostate cancer cells results in increase in growth rate and migration and tumor cell colony formation. Interestingly, the data show that the human SRD5A2 gene is at least as potent and efficacious. In addition, this experimental finding is also strongly supported by the association between lack of SRD5A2 expression and increased tumor cell proliferation in clinical specimens on human prostate tissue microarray. Finally, analysis of SRD5A2 protein expression in the prostate tumor microarray indicates that SRD5A2 is selectively reduced in metastases and in locally recurrent hormone insensitive tumors. Taken together, these results strongly suggest that SRD5A2 may act as a TSG, not only in prostate cancer cell lines, but also in clinical prostate cancer, where loss of SRD5A2 expression appears to be highly correlated with late-stage prostate cancer. Thus, compared with other TSGs like PTEN yMMAC1 in prostate cancer, reduced expression of the SRD5A2 gene is strongly correlated with the most clinically compromising forms of this cancer. Future large-scale studies on tissue microarrays hopefully will elucidate the correlation among these multiple molecular markers with prostate cancer progression and comprehensively illuminate the complex relationships among these genes.

In clinical studies it has been observed that SRD5A1 and SRD5A2 mRNA were present at a much higher levels in localized high vs low-grade prostate cancer whereas 5alpha-reductase type 1 appeared to be higher in benign tissue adjacent to cancer than in benign prostatic hyperplasia.[129]. The higher fetal SRD5A2 expressions in the epithelium cells than in the stroma cells is significant with respect to the prostate cancer. These
observations are corroborated by findings of other groups showing increased SRD5A1 activity but with declining SRD5A2 activity during prostate cancer development. Results suggested that both the isoenzymes may participate in the pathophysiology of prostate cancer [130] and it has been postulated that cutting DHT level with enzyme inhibitors might have some therapeutic implication [49].

Based on this hypothesis, few drugs have been developed to have inhibitory effect on 5-alpha reductase enzyme activity and have been approved by the FDA for clinical use. They are now being used in the clinic but their efficacy in disease control remains controversial. Data on the level of SRD5A2 gene product in prostate cancer patients are ambiguous and therefore, correlation between pathogenic condition and SRD5A2 levels remains elusive. The direction and extent of changes in SRD5A2 expression in disease tissues is a relevant issue in this regard. A number of published data indicated an association of prostate cancer with reduced 5alpha-reductase enzymatic activity as a result of remarkably decreased expression of the SRD5A2 gene [131] while others have reported increased expression of androgen converting enzymes in androgen independent metastatic tumor samples [132] These result suggests that androgen independent prostate cancer will require novel agents capable of inhibiting intracrine steroidogenic pathways within the prostate tumor microenvironment. Therefore, our work is to understand the exact role of SRD5A2 gene in the cell growth and apoptosis in prostate cancer.

**Proposed Functional model for SRD5A2**

SRD5A2 function was analysed at different level using antisense inhibition as well as Inhibition of protein function by small molecule. We have proposed a model for the function of SRD5A2. In normal function, testosterone is converted in Dihydrotestosterone, which further induces changes in the prostate cells. T conversion to DHT regulated by SRD5A2 enzyme. We have seen that when small molecule FZ is used to treat prostate cancer cell it reduced cell growth. As we have seen that FZ is superimposed with testosterone and because of this it will not be able to bind at the binding
site at SRD5A2 protein as studied by bioinformatics studies. Which in turn decrease substrate for the conversion of DHT and result in the reduced activity of SRD5A2. Secondly, we have used antisense clone against the mRNA of SRD5A2 that result in decrease of the SRD5A2 protein and could able to converted T to DHT. Both the approach results in the downregulation of DHT which further result in the decrease in the cellular growth of prostate cancer and tumor growth in nude mice experiments. The reduced cell growth inhibition result in the increase sub G1 population and DNA fragmentation that induced the mitochondrial dependent apoptosis in the prostate cancer cells. Down regulation of DHT is also a result in the increase of Cell migration, invasion, colony formation and cellular growth. In the genetic manipulation of SRD5A2, the cellular growth is increased in contrast to small molecule inhibition. The pathways that trigger in individual case could be different and result in the contrast result. At last, SRD5A2 protein is important for maintaining cell growth and migration in prostate cancer cells.

Figure 62: Proposed model for the role SRD5A2 prostate cancer cell growth and apoptosis.
Finally, we identified a benzimidazole compound, methyl N-(6-phenylsulfanyl-1H-benzimidazol-2-yl) carbamate (FZ), that effectively inhibited the growth of normal human and mouse fibroblasts at the IC50 values used for prostate cancer cells, suggesting that FZ might selectively induce cytotoxicity in human prostate cancer cells. Although p53 has a crucial role in cell cycle regulation and apoptosis induction in various types of cancer cells after treatment with chemotherapeutic agents, FZ effectively inhibited cell growth in both PC 3 and Dul 45 cell lines independent of p53 status. Consistently, previous report has shown that PC3 and DU 145 cell cells had a very low level of SRD5A2 expression and were relatively resistant to paclitaxel in vitro. Notably, there cells were sensitive to FZ while normal cells were spared. Moreover, FZ inhibited the growth of human xenograft in nu/nu mouse model in vivo without any toxicity to the normal untreated animals.

Although most antineoplastic agents induce apoptosis in cancer cells, the mechanisms by which they do so remain unclear. In this study, we showed that caspase activation was involved in FZ induced apoptosis, as is the case with numerous chemotherapeutic agents.

The tumor-selective cytotoxic effect induced by FZ might be useful in cancer therapy. However, the mechanism of selectivity of the compound and initial targets are not yet known. The selective cell killing observed may have been the result of cellular differences in drug uptake and metabolism or biochemical and physiologic differences in activation, transduction, and duration or various signaling pathways. To determine whether FZ and its analogues can be used or treat human colorectal cancers, we will need to further characterize the molecular mechanisms of the compounds and screen for analogues with better efficacy and selectivity and acceptable in vivo pharmacokinetic properties. Our identification and initial characterization of apoptosis induction by FZ is only the first step in developing new anticancer agents.