Differential gene expression patterns in genital fibroblasts of normal males and 46, XY-females with androgen insensitivity syndrome:

*Evidence for early programming involving the androgen receptor*

**Running title:** Gene expression in genital fibroblasts

**Authors:** ¹²P. M. Holterhus, ²O. Hiort, ³J. Demeter, ⁴P. O. Brown, and ¹J. D. Brooks

**Corresponding authors:**
J.D.B. (jbrooks1@leland.Stanford.EDU; phone: 650 725 5544; fax: 650 723 0765)
P.M.H. (holterhus@paedia.ukl.mu-luebeck.de)
P.O.B.(pbrown@cmgm.stanford.edu)

**Addresses:** ¹Department of Urology, Stanford University School of Medicine, Stanford, California, 94305, USA; ²Department of Pediatrics, University of Lübeck, 23538 Lübeck, Germany; ³Department of Genetics, Stanford University School of Medicine, Stanford, California, 94305, USA; ⁴Department of Biochemistry and the Howard Hughes Medical Institute, Stanford University School of Medicine, Stanford, California, 94305, USA

**Key words:** male pseudohermaphroditism, androgen insensitivity syndrome, androgen receptor, genital fibroblast, gene expression, microarray

**Abbreviations:** AIS = androgen insensitivity syndrome, AR = androgen receptor, DHT = dihydrotestosterone, SAM = significance analysis of microarrays
Abstract

Androgen insensitivity syndrome (AIS) comprises a range of phenotypes from male infertility to complete feminization. Most individuals with AIS carry germ-line mutations of the androgen receptor (AR) that interfere with or ablate its function. Since genital fibroblasts retain expression of the AR in vitro, we used genital skin fibroblasts from normal males and 46,XY females with complete AIS and known AR-mutations to gain insights into the role of the AR in human genital differentiation. Using DNA microarrays representing 32,968 different genes we identified 404 transcripts with significant differences in expression between genital skin fibroblasts cultured from normal and AIS-affected individuals. Gene cluster analyses uncovered coordinated expression of genes involved in key processes of morphogenesis. Several of these genes are known to have specific roles in genital differentiation, based on animal studies and human genetic syndromes. Remarkably, genital fibroblasts from both normal and AIS-affected individuals showed no transcriptional response to high-dose dihydrotestosterone treatment despite documented expression of the AR. The results suggest that androgen signaling during prenatal development is involved in setting long-lasting, androgen-independent transcriptional programs in these cells. Our findings have broad implications in understanding the establishment and stability of sexual dimorphism in human genital development.
Introduction

Development of the male genitalia is largely controlled by androgen receptor (AR) expressing cells in the urogenital mesenchyme (Cunha et al., 1980; Taakeda et al. 1985). Germ-line mutations of the AR gene produce a spectrum of developmental abnormalities in 46, XY individuals ranging from infertility or mild hypospadias to complete feminization, which are collectively referred to as the androgen insensitivity syndrome (AIS). In general, the degree of genital ambiguity correlates with the level of compromise of AR function: 46,XY individuals with AR-inactivating mutations are completely feminized despite high serum testosterone levels (McPhaul et al., 1993; Quigley et al. 1995; Hiort et al. 1998).

The molecular events responsible for AR-dependent male genital morphogenesis are poorly understood. We hypothesized that the AR-dependent mesenchymal programs underlying male external genitalia development might be illuminated by comparing the transcriptional profile of mesenchymal cells from normal males to those from individuals affected with AIS. Since cultured genital fibroblasts are derived from urogenital mesenchyme and retain expression of the AR in vitro, we compared gene expression patterns in cultured genital fibroblasts from normal 46, XY males and from 46, XY females with severe or complete AIS, using DNA microarrays representing 32,968 distinct human genes. We found striking differences in the gene expression profiles of genital fibroblasts cultured from normal and AIS patients, but no transcriptional response to androgen was detectable in any of the cultured genital fibroblasts.
Methods

The study was approved by the ethical committee of the Medical University of Lübeck, Germany. Informed consent was obtained from all normal subjects and AIS-patients or their parents.

Cell strains

Primary cultures of genital fibroblasts were established from genital skin biopsies (labia majora) or gonadal biopsies in female AIS-patients and from the foreskin of normal males undergoing circumcision. Abdominal skin fibroblasts were derived from the midline above the mons pubis of a fertile male during abdominal surgery. Forearm skin fibroblasts from a normal male were a gift from H. Chang (Dept. of Biochemistry, Stanford University). Peripheral zone prostate fibroblasts were a gift from D. Peehl (Dept. of Urology, Stanford University) and were established from a histologically normal region of a patient undergoing prostatectomy for prostate cancer who had not been previously treated with hormonal therapy. Hormone binding assays and androgen receptor sequencing have been described previously (Holterhus et al., 1997).

Cell culture and hormone treatment

For determination of basal gene expression profiles without androgen stimulation, fibroblasts were cultured on 150mm plastic dishes at 37°C with 5% CO₂. To eliminate possible artifacts due to differing states of proliferation, cells were grown to confluency wherein they enter G₀ arrest (Tobey et al., 1988). They were maintained in phenol-red-free DMEM F12
Holterhus et al., Gene transcription in genital fibroblasts

(Dulbecco’s modified Eagle Medium with the nutrient mix F12, Gibco) containing L-glutamine, 15mM Hepes buffer, penicillin / streptomycin (Gibco) and 12.9% of a constant lot of certified fetal calf serum (FCS, Gibco). The pH was adjusted to 7.4 with 1N NaOH and the media was exchanged every 48h. At day 13, the last media exchange was performed and 96h later cells were scraped and mRNA harvested directly.

Androgen stimulation of genital fibroblasts was carried out under two different culture conditions similar to those previously reported to produce induction of aromatase enzymatic activity in these cell lines (Chabab et al., 1986; Stillmann et al., 1991). In the first, cells were grown to confluency as described above using phenol-red-free DMEM F12 containing L-glutamine, 15mM Hepes buffer, penicillin / streptomycin (Gibco) with 12.9% charcoal stripped, steroid free FCS (D/S-FCS) (Hyclone) to ensure androgen depleted conditions in control cells. With every media exchange, cells received either ETOH in a final dilution of 1:100,000 or 100nM dihydrotestosterone (DHT) dissolved in ETOH. The last DHT treatment was administered with the last media exchange 96 hours prior to lysate preparation. In total, 6 doses of either ETOH or 100nM DHT were given.

In the second set of experiments, cells were cultured to confluency for 14 days as described above. They were then trypsinized and seeded at a density of 3000 cells per cm² in 150 mm plates. 24h later, media was removed, and cells were washed 3 times with new media containing 12.9% D/S-FCS, then cultured for another 24h interval in the absence of androgens. Cells were then treated with either 1:100,000 ETOH, 100nM or 1000nM DHT dissolved in ETOH. After 24h incubation, exponentially growing cells were harvested.
RNA-isolation and cDNA-labeling

Protocols for mRNA preparation and cDNA labeling are available at http://cmgm.stanford.edu/pbrown. 2 μg of mRNA from single experiments was reverse transcribed and labeled with Cy5 (pseudo-coloured red) and pooled reference mRNA was labeled with Cy3 (pseudo-coloured green). Reference mRNA contained equal mixtures of fibroblast mRNA (pooled from confluent and proliferating cultures of normal and AIS genital skin fibroblasts) and a “common reference” of mRNAs isolated from 11 different proliferating cultured tumor cell lines we have described previously (Perou et al., 2000).

Microarrays and hybridizations

Microarrays with approximately 43,000 sequence-validated PCR-amplified human cDNAs representing 32,968 UniGene clusters were manufactured as described (http://cmgm.stanford.edu/pbrown). Hybridizations were performed using equal amounts of Cy3 and Cy5 labeled cDNAs according to previously published protocols (http://cmgm.stanford.edu/pbrown). Hybridized microarrays were scanned using a GenePix4000 array scanner and analyzed with GenePix Pro 3.0 software (Axon Instruments, Inc., Union City, CA).

Microarray data analysis

Only spots with fluorescence signals 1.5-fold over background in either the experimental or reference samples were included in the analysis. To correct for variations in cDNA labeling efficiency, we normalized the Cy5 / Cy3 fluorescence ratios for all genes in each array hybridization to obtain an average log₂ (ratio) of 0. We restricted our analysis to genes with
measurable expression in 80% of the samples we analyzed. To identify genes with statistically significant differences in baseline expression levels between normal and AIS genital fibroblasts, we used the Significance Analysis of Microarrays (SAM) procedure (Tusher et al., 2001). The SAM procedure computes a two-sample T-statistic (e.g. for normal vs. AIS cell lines) for the normalized log ratios of gene expression levels for each gene. It thresholds the T-statistics to provide a “significant” gene list and provides an estimate of the false discovery rate (the percent of genes identified by chance alone) from randomly permuted data.

To identify the effects of androgen treatment on gene expression in genital fibroblasts, we carried out a set of 21 DNA microarray analyses of RNA from normal and AIS genital fibroblasts. This data set included cells treated at confluence ($G_0$) or during exponential proliferation as described above. Raw data were filtered, normalized and centered as described above. We used the SAM procedure to identify transcripts with significant differences in expression based on origin of the fibroblast lines, whether the cells were confluent or proliferating, and whether they had been treated with androgen.

Results

Analysis of baseline gene expression in genital fibroblasts.

To gain insights into the role of androgen in genital morphogenesis, we compared basal transcriptional patterns in genital fibroblasts from 46, XY individuals with either wild-type AR or germ-line inactivation of AR due to mutation. We could not identify AR gene mutations in two females with complete AIS. However, genital skin fibroblasts of both subjects failed to
express AR protein and did not show androgen binding (table 1). Initially, we restricted our analysis to genital skin fibroblasts grown from the foreskin of 9 normal males and from the labia majora of 5 AIS-affected females. AR status of all fibroblasts was confirmed by AR gene sequencing and hormone binding assays (table 1). To determine basal gene expression patterns, mRNA was isolated from growth-arrested (G0) confluent cells and analyzed using DNA microarrays of approximately 43,000 cDNAs representing 32,968 genes. Distinct differences in the basal expression profiles of normal and AIS derived fibroblasts allowed these two groups to be distinguished on the basis of their expression patterns by unsupervised hierarchical clustering analysis (Fig. 1). We then identified 404 unique transcripts (represented by 487 total cDNAs) with significant differences in expression levels between normal and AIS genital fibroblasts using the Significance Analysis of Microarrays (SAM)-procedure (Tusher et al. 2001), with a false discovery rate of < 0.92% (percent of genes identified by chance alone).

We used the list of the 487 cDNAs from the SAM analysis for hierarchical clustering analysis of 24 different primary fibroblast lines from normal and AIS affected individuals (Fig. 2). In addition to the 14 genital skin fibroblast lines used to generate the SAM list, we included 5 gonadal fibroblast lines from 46,XY females with complete AIS, a prostate fibroblast cell line from a normal male (analyzed twice), abdominal skin fibroblasts from a normal male, and forearm fibroblasts from a normal male, and genital fibroblasts from two AIS 46, XY females who had documented AR mosaicism (ARD364, ARD 465, table 1). The ARD364 and ARD465 lines express wild-type AR and were derived from individuals who were mosaics for wild-type AR and AR with a premature stop codon (Holterhus et al., 1997; table 1). Hierarchical cluster analysis, based on the expression of 472 of the 487 previously identified transcripts
with measurable expression across at least 80% of 24 experiments, separated the fibroblasts into those with gene expression patterns resembling normal male foreskin fibroblasts and a second group with an expression pattern more similar to labial skin fibroblasts from AIS-affected individuals (Fig. 2). The latter group contained all 5 gonadal fibroblast lines from complete AIS females as well as the fibroblast lines from abdominal and forearm skin. The prostate fibroblasts, on the other hand, displayed expression patterns largely similar to the normal male foreskin cells. Notably, the two mosaic AIS cell lines ARD364 and ARD 465 showed gene expression patterns that most resembled normal male foreskin (Fig. 2).

Comparison of expression patterns in fibroblasts from normal and AIS-affected individuals, and fibroblasts from extragenital sites offers possible insights into the programs that underlie genital development. For instance, transcripts encoding homeobox A13 protein and T-box proteins showed striking differences in their expression levels between the “male genital” and “AIS/extragenital” fibroblasts. HOXA13 was expressed at high levels in normal male foreskin fibroblasts and at low levels in all AIS and extragenital fibroblasts (Fig. 2). T-box gene 3 (TBX3) was expressed at higher levels in the fibroblasts from genital skin, extragenital skin or prostate from males than in genital skin fibroblasts from AIS 46, XY females (Fig. 2). TBX2 showed an almost identical expression profile to TBX3, while high TBX5 expression appeared to be restricted to foreskin fibroblasts from normal males (including those from the phenotypically female mosaic patient ARD364). BMP4 was predominantly expressed in foreskin fibroblasts from normal males and in prostate fibroblasts (Fig. 2). WNT2 was part of a small gene cluster with high expression in normal male foreskin fibroblasts that distinguished these samples from all other fibroblasts (Fig. 2).
Compared to genital and gonadal fibroblasts from 46,XY females, fibroblasts from normal male genital tissues showed pronounced differences in expression of cell adhesion and extracellular matrix genes. For example, cadherin 13 (CDH13), versican (CSPG2), collagen 8A1 (COL8A1), collagen 12A1 (COL12A1), P4HA2 (a procollagen-modifying enzyme) all showed relatively low expression in the genital skin fibroblasts of normal males, whereas tenascin XB (TNXB), nidogen 2 (NID2), laminins (LAMA3, LAMA4, LAMC1), and tissue inhibitor of metalloproteinase 1 (TIMP1) all showed relatively high expression levels compared to AIS-derived fibroblasts (Fig. 2). Several of the differentially expressed genes, including aldo-keto reductase 1C1 (AKR1C1), aldehyde dehydrogenase 1A1 (ALDH1A1), and alcohol dehydrogenase 1B (ADH1B), function in sex steroid and retinoic acid metabolism (Fig. 2). Other differentially expressed genes, such as mitogen activated protein kinases 14 (MAP3K14), and STAT induced STAT inhibitors 2 and 3 (STATI2, SSI-3), encode proteins involved in intracellular signal transduction.

**Transcriptional response of cultured genital fibroblasts to androgen treatment**

We tested the responses of normal and AIS genital fibroblasts to DHT, under culture conditions nearly identical to those that were reported to produce aromatase induction in these cells (Stillmann et al., 1991). Cells were treated with DHT (100-1000nM) both at confluency (G₀) and during exponential growth, and transcript levels were assessed using DNA microarrays. Hierarchical clustering did not disclose any obvious differences in gene expression patterns between DHT-treated cells and ethanol treated controls. A supervised analysis comparing gene expression patterns of DHT-treated fibroblasts to ethanol-treated controls was carried
out using the SAM procedure. Again, no genes could be identified that were significantly induced or repressed by DHT treatment. In contrast, SAM-analysis identified 1664 transcripts that differed significantly between proliferating and confluent cells, and 1232 transcripts that differed between fibroblasts derived from AIS-affected individuals and normal male foreskin. Hierarchical cluster analysis of these experiments clearly showed the distinct differences in transcriptional profiles between AIS-derived and normal male fibroblasts and between proliferating and normal fibroblasts (Fig. 3). Cells derived from the same individual and cultured under the same conditions always showed highly similar gene expression patterns, suggesting that the differences in expression between individuals are stable and reproducible despite passage in vitro (Fig. 3). On the other hand, hierarchical cluster analysis could not distinguish between DHT-treated and untreated cells, in either normal or AIS-derived fibroblasts. Transcriptional profiles in each cell lines appeared unaffected by androgen treatment or deprivation. In addition, treatment of normal and AIS-derived genital fibroblasts with the stable androgen analogue methyltrienolone (R1881) under several conditions failed to produce changes in gene expression (data not shown).

**Discussion**

We found consistent, characteristic differences in baseline gene expression patterns between genital skin fibroblasts from normal males and 46, XY female patients with AIS. Many of these differences between normal and AIS derived fibroblasts were also observed in gonadal fibroblasts, suggesting these differences are not purely due to differences in the anatomical site of origin of the fibroblasts. Interestingly, fibroblasts derived from abdominal and forearm skin, regions with relatively little sexual dimorphism, showed gene expression patterns similar to the
labial skin fibroblasts of AIS patients. Together, these data suggest that the AR is involved in determining a stable and stereotypical program of gene expression in genital fibroblasts that does not require the continuing presence of androgen for its maintenance.

A critical question raised by these results is whether the observed differences between genital fibroblasts from males and AIS females reflect cell-autonomous effects of androgen exposure during development or indirect effects of the AR-dependent genital morphogenic program. One possible interpretation of these data is that the distinct patterns of expression could have been due to differences in the origin or the developmental milieu of foreskin fibroblasts, derived from the genital tubercle, as opposed to the labial fibroblasts, derived from the genital swellings (Rey and Picard, 1998). The differences in gene expression we observed in AIS fibroblasts of gonadal origin compared to those of labial origin supports this view (Fig. 3). We have observed consistent and characteristic differences in the gene expression patterns of skin fibroblasts derived from different locations on the body (Chang et al. 2002). However, the current set of experiments strongly suggests that the androgen receptor has a cell-autonomous role in determining a stable androgen-independent gene expression pattern in genital fibroblasts. Expression patterns in cultured labial skin fibroblasts derived from two different individuals with AR mosaicism suggested that the cell-autonomous AR status was a major determinant of baseline gene expression in genital skin fibroblasts. Both of these fibroblast lines, although derived from morphologically female genitalia in phenotypically female 46, XY individuals mosaic for AR-inactivating mutations, expressed wild-type AR in the cultured cells. These female AIS-affected individuals are thought to have acquired their AR gene mutations post-zygotically (Holterhus et al., 2001). ARD364, which showed AR protein expression and binding in the range of normal male foreskin fibroblasts (Holterhus et al., 1997; table 1), de-
spite its origin from anatomically female genitalia, had a gene expression pattern indistinguishable from foreskin fibroblasts of normal males (Fig. 2). The second fibroblast line from an AR mosaic patient, ARD465 (table 1), had lower wild-type AR-expression and showed baseline gene expression patterns that were nevertheless more similar to normal male foreskin and prostate fibroblasts than to any of the AIS-derived cell lines (Fig. 2). Together these data suggest that the AR-status of the fibroblast itself, rather than the genital structure from which the fibroblasts are derived, was the major determinant of the basal expression patterns of the transcripts we identified. Therefore, AR appears to be involved in setting long-lasting gene expression patterns in genital skin fibroblasts.

Comparison of gene expression patterns in genital fibroblasts from normal and AIS-affected individuals, and fibroblasts from extragenital sites may offer clues to the programs that underlie external genital development. Both cell adhesion and connective tissue remodeling are indispensable for normal development and maintenance of tissue integrity (Gumbiner, 1996; McNeil, 2000; Woods, 2001). The differential expression of proteoglycans, collagens and cell adhesion molecules (e.g., cadherin 13) might be involved in genital morphogenesis and later stability of sexually dimorphic traits of the external genitalia. Some genes expressed in wild-type AR cells could influence androgen signaling. For instance, aldo-keto reductase 1C1 is specifically involved in cellular androgen metabolism (Penning et al., 2000) and thus may modulate the spectrum of cellular androgenic steroids available for activation of the AR. Structurally different androgens elicit different patterns of response from several androgen-responsive promoters, suggesting that the type of ligand present could affect cellular response (Holterhus et al. 2002, in press). Mitogen-activated protein kinase 14, and STAT induced
STAT inhibitors 2 and 3 were expressed at significantly higher levels in cells with wild-type AR. Both MAPK - and STAT - pathways are involved in AR-dependent regulation and in ligand-independent activation of the AR (Ueda et al., 2002). Differential expression of aldehyde dehydrogenase 1A1 and alcohol dehydrogenase 1B, enzymes that affect retinoic acid biosynthesis, suggest that other signaling pathways may participate in the AR-initiated programs of external genital differentiation (Vonesch et al., 1994, Ang and Duester, 1997).

Several genes expressed specifically in the normal male foreskin fibroblasts have been previously implicated in male genital development, including HOXA13, the T-box genes, BMP4 and D-Wnt2. HOXA13-mutations can cause distal limb and genitourinary tract malformations such as male hypospadias in hand-foot-genital syndrome (Mortlock and Innis, 1997). T-box (TBX) genes are essential early regulators of limb development and also appear to be involved in male genital development (Rodriguez-Esteban et al. 1999; Bamshad et al., 1997). Mutations in TBX3 cause the ulnar-mammary syndrome characterized by limb, apocrine, and genital developmental abnormalities (Bamshad et al., 1997). Expression of T-box genes 2, 3, and 5 was higher in normal male foreskin fibroblasts than in AIS genital fibroblasts. BMP4 has been implicated in ductal budding and branching during prostate development (Lamm et al. 2001) and a potential role of BMP4 in external genital development has also been postulated (Haraguchi et al. 2001). D-Wnt2 has been found to play roles in sex-specific cell determination in the gonads and genital disc of Drosophila (Kozopas et al., 1998). Thus mutations in genes characteristically expressed in normal male foreskin fibroblasts can, in some cases, lead to defective genital development. The data from these experiments therefore provide candidate genes for further investigation in patients with genital malformations.
Since normal genital skin fibroblasts of 46, XY male individuals express the AR in vitro (Quigley et al., 1995, Holterhus et al., 1997), we had anticipated that androgen treatment would elicit a transcriptional response program that could provide additional insights into the role of androgen in genital development. Furthermore, we hoped that comparison of transcriptional responses of normal fibroblasts to AIS-affected individuals with varying degrees of genital ambiguity would provide still further insights into androgen’s role in genital morphogenesis. Two previous reports have shown considerable increases in aromatase enzymatic activity in genital skin fibroblasts treated with dihydrotestosterone (DHT) (Chabab et al. 1986, Stillmann et al., 1991). Moreover, a previous study of the transcriptional response of prostate cancer cells to androgen had revealed consistent global changes in gene expression. However, we were unable to detect any significant changes in gene expression patterns in cultured, AR-expressing genital fibroblasts in response to androgens.

Existing literature on androgen-regulated gene transcription in genital fibroblasts is sparse and contradictory (Stillmann et al., 1991, Berkovitz et al., 1990, Nitsche et al., 1996, Elmlinger et al., 2001). The apparent insensitivity of normal genital skin fibroblasts to DHT in vitro in our experiments was in sharp contrast to the robust transcriptional response program we have observed in the human prostate cancer cell line LNCaP (DePrimo et al., 2002). AR-expression itself may be insufficient in genital skin fibroblasts to elicit a transcriptional response. Indeed, in normal genital fibroblasts, androgen responsive reporter genes can only be activated by high level expression of co-transfected AR in the presence of ligand (Holterhus et al., 2002). Comparison of basal gene expression of normal male genital fibroblasts with that in
LNCaP cells demonstrated diminished expression of several AR co-regulators in genital fibroblasts (e.g., NCOA2 (GRIP-1), NCOA3 (TRAM-1), ARA54 (RNF14), data not shown), suggesting importance of a distinct spectrum of AR co-regulators. It is possible that genital mesenchymal cells are only capable of responding to androgen at discrete stages in development in their specific in-vivo environment, and this responsiveness may be mediated through the expression of specific AR co-regulators. Prostate epithelial cells retain exquisite sensitivity to androgen throughout life. Androgen deprivation produces profound involution of the prostate, particularly of the epithelial component, but little change in the external genitalia. Therefore, the differences in responsiveness to androgen seen in prostate and genital fibroblasts in vitro may reflect the pattern of responsiveness in vivo.

Our work suggests that in addition to androgen-independent positional influences on fibroblast phenotypes, the AR is originally involved in establishing stable and reproducible patterns of gene expression in stromal cells during genital differentiation, which are reflected in the differences in global gene expression patterns between fibroblasts cultured from the genital skin of normal individuals and females affected by AIS. Comparison of the expression patterns of genital fibroblasts from 46,XY normal males and 46,XY females with inactivated AR provides a window on the AR-dependent gene expression programs within the urogenital mesenchyme, which contribute to the development and structural integrity of male and female genitalia. The apparent lack of response of genital fibroblasts to androgen in vitro, despite expression of a normal AR, has important implications for future research in defining the role of androgen in genital development and the pathogenesis of ambiguous genitalia. Transcriptional
profiling of the early stages genital development in vivo in the presence and absence of androgen may provide further insights into the role of androgen in genital development.

Acknowledgements

The study was supported by a grant from the National Cancer Institute (P.O.B.), the Howard Hughes Medical Institute (P.O.B.), the Deutsche Forschungsgemeinschaft (DFG) (grants Ho 2073 / 2-1, 2-2 and KFO 111 / 1-C to P.M.H.) and the Doris Duke Charitable Foundation (J.D.B.). We thank Genevieve Vidanes, Nicole Homburg, Christine Marschke, and Dagmar Struve for excellent technical assistance, Michael Whitfield and Samuel DePrimo for expert advice, and Rob Tibshirani, Orly Alter and Jonathan Pollack for discussions of microarray data analysis. We thank the scientists and staff of the Stanford Microarray Facility and the Stanford Microarray Database. We also thank all physicians and members of the German Collaborative Intersex Study Group for contributing genital fibroblast samples and clinical information, especially Drs. Albers, Brämswig, Bull, Hoepffner, Jocham, Kleinkauf-Houcken, Korsch, Schwarz, Schölermann and Wollmann. P.O.B. is an Investigator of the Howard Hughes Medical Institute.

Figures and table

Figure 1:

Unsupervised hierarchical cluster analysis of genes and experiments of 9 normal genital skin fibroblast lines (penile foreskin) and 5 AIS genital skin fibroblast lines (labia majora). Only transcripts whose log₂ red / green ratio differed from the mean expression level across all ex-
periments by at least 1.1 in at least three experiments are displayed (620 cDNAs). The dendrogram of the array experiments reflects the similarity of the samples with respect to their gene expression patterns. $F = \text{female}, M = \text{male}, \text{NORM} = \text{normal male control}, \text{AIS4} = \text{AIS with predominantly female phenotype}, \text{CAIS} = \text{complete androgen insensitivity syndrome}$. Increasing red intensity corresponds to increased gene expression levels compared to the mean log$_2$ red/green ratio for each gene; increasing green intensity corresponds to decreased gene expression levels. The complete dataset is available at http://genome-www5.stanford.edu/cgi-bin/SMD/publication/viewPublication.XYZ

**Figure 2:**
Hierarchical cluster analysis of genes and experiments based on cDNAs identified as being significantly different in expression between normal genital skin fibroblasts and genital skin fibroblasts of female patients with AIS. The left panel shows an overview of 472 of the total of 487 significant transcripts that showed measurable expression across at least 80% of 24 experiments. The color code of the dendrogram and the sample names represent the origin of the fibroblast strains. The complete dataset is available at http://genome-www5.stanford.edu/cgi-bin/SMD/publication/viewPublication.XYZ

**Figure 3:**
Hierarchical cluster analysis of genes and experiments with different DHT treatment regimens. Shown are the 2862 transcripts that distinguish between normal genital skin fibroblasts and gonadal fibroblasts from female AIS patients, and between proliferating and confluent fibro-
blasts. The color code in the dendrogram depicts the origin of the fibroblast cultures. The gray and white bars on top of the cluster indicate the proliferation state of the samples. On the right, the regions of the cluster diagram are indicated which differentiate between normal and AIS-derived fibroblasts, and proliferating and confluent cells, respectively. No differences in transcript levels could be discerned between DHT treated and control cells in either normal foreskin fibroblasts or fibroblasts from AIS affected females. The complete dataset is available at http://genome-www5.stanford.edu/cgi-bin/SMD/publication/viewPublication.XYZ.

Table 1:

GSF = genital skin fibroblast, GOF = gonadal fibroblast, PRF = prostate fibroblast, ASF = abdominal skin fibroblast, FSF = forearm skin fibroblast, ARD = strain-ID of the Androgen Receptor Database of the German Collaborative Intersex Study group in Lübeck, Germany.

References

- Ang, H.L., and Duester, G. (1997). Initiation of retinoid signaling in primitive streak mouse embryos: spatiotemporal expression patterns of receptors and metabolic enzymes for ligand synthesis. Dev. Dyn. 208, 536-543
- Bamshad, M., et al. (1997). Mutations in human TBX3 alter limb, apocrine and genital development in ulnar-mammary syndrome. Nat. Genet. 16, 311-315
- Berkovitz, G.D., Carter, K.M., Brown, T.R., and Migeon, C.J. (1990). Testosterone lowers aromatase activity in cultured human genital skin fibroblasts. Mol. Cell. Endocrinol. 69, 187-197
- Chabab, A., Sultan, C., Fenart, O., and Descomps, B. (1986). Stimulation of aromatase activity by dihydrotestosterone in human skin fibroblasts. J. Steroid Biochem. 25, 165-169
- Cunha, G.R., Chung, L.W.K., Shannon, J.M., and Reese, B.A. (1980). Stromal-epithelial interactions in sex differentiation. Biol. Reprod. 22, 19-42
- DePrimo, S.E., Diehn, M., Nelson, J.B., Reiter, R.E., Matese, J., Fero, M., Tibshirani, R., Brown, P.O., and Brooks, J.D. (2002). Transcriptional programs activated by exposure of human prostate cancer cells to androgen. Genome Biol. 3, research0032.1-0032.12
• Eisen, M.B., Spellmann, P.T., Brown, P.O., and Botstein, D. (1998) Cluster analysis and display of genome-wide expression patterns. Proc. Natl. Acad. Sci. USA 95, 14863-14868
• Elmlinger, M.W., et al. (2001). Decreased expression of IGF-II and its binding protein, IGF-binding protein-2, in genital skin fibroblasts of patients with complete androgen insensitivity syndrome compared with normally virilized males. J. Clin. Endocrinol. Metab. 86, 4741-4746
• Gumbiner B.M. (1996). Cell adhesion: the molecular basis of tissue architecture and morphogenesis. Cell 84, 345-357
• Haraguchi, R., Mo, R., Hui, C., Motoyama, J., Makino, S., Shiroishi, T., Gaffield, W., and Yamada, G. (2001). Unique functions of Sonic hedgehog signaling during external genitalia development. Development 128, 4241-4250
• Hiort, O., Holterhus, P.M., and Nitsche, E.M. (1998). Physiology and pathophysiology of androgen action. Baillières Clin. Endocrinol. Metab. 12, 115-132
• Holterhus P.M., Brüggenwirth H.T., Brinkmann A.O., and Hiort O. (2001). Post-zygotic mutations and somatic mosaicism in androgen insensitivity syndrome. Trends Genet. 17, 627-628
• Holterhus, P.M., Brüggenwirth, H.T., Hiort, O., Kleinkauf-Houcken, A., Kruse, K., Sinner, G.H., and Brinkmann, A.O. (1997). Mosaicism due to a somatic mutation of the androgen receptor gene determines phenotype in androgen insensitivity syndrome. J. Clin. Endocrinol. Metab. 82, 3584-3589
• Holterhus, P.M., Piefke S., and Hiort O. (2002). Anabolic steroids, testosterone-precursors and virilizing androgens induce distinct activation profiles of androgen responsive promoter constructs. J Steroid Biochem Mol Biol (in press)
• Holterhus, P.M., Salzburg, J., and Hiort O. (2002). Transactivation properties of natural androgen receptor gene mutations in transfected human genital fibroblasts. Horm Res. 58, Suppl. 2
• Kozopas, K.M., Samos, C.H., and Nusse, R. (1998). DWnt-2, a Drosophila Wnt gene required for the development of the male reproductive tract, specifies a sexually dimorphic cell fate. Genes Dev. 12, 1155-1165
• Lamm, M.L., Podlasek, C.A., Barnett, D.H., Lee, J., Clemens, J.Q., Hebner, C.M., and Bushman, W. (2001). Mesenchymal factor bone morphogenetic protein 4 restricts ductal budding and branching morphogenesis in the developing prostate. Dev. Biol. 232, 301-314
• Lim, L., Kalinichenko, V.V., Whitsett, J.A., and Costa, R.H. (2002). Fusion of lung lobes and vessels in mouse embryos heterozygous for the forkhead box f1 targeted allele. Am. J. Physiol. Lung Cell. Mol. Physiol. 282, L1012-1022
• McNeill, H. (2000). Sticking together and sorting things out: adhesion as a force in development. Nat. Rev. Genet. 1, 100-108
• McPhaul, M.J., Marcelli, M., Zoppi, S., Griffin, J.E., and Wilson, J.D. (1993). Genetic basis of endocrine disease. 4. The spectrum of mutations in the androgen receptor gene that causes androgen resistance. J. Clin. Endocrinol. Metab. 76, 17-23
• McPhaul, M.J., Schweikert, H.J., and Allman, D.R. (1997). Assessment of androgen receptor function in genital skin fibroblasts using a recombinant adenovirus to deliver an androgen-responsive reporter gene. J. Clin. Endocrinol. Metab. 82, 1944-1948
• Mortlock, D.P., and Innis J.W. (1997). Mutation of HOXA13 in hand-foot-genital syndrome. Nat. Genet. 15, 179-180
• Nitsche, E.M., Moquin, A., Adams, P.S., Guenette, R.S., Lakins, J.N., Sinnecker, G.H., Kruse, K., and Tenniswood, M.P. (1996). Differential display RT PCR of total RNA from human foreskin fibroblasts for investigation of androgen-dependent gene expression. Am. J. Med. Genet. 63, 231-238
• Penning, T.M., Burczynski, M.E., Jez, J.M., Hung, C.F., Lin, H.K., Ma, H., Moore, M., Palackal, N., and Ratnam, K. (2000). Human 3alpha-hydroxysteroid dehydrogenase isoforms (AKR1C1-AKR1C4) of the aldo-keto reductase superfamily: functional plasticity and tissue distribution reveals roles in the inactivation and formation of male and female sex hormones. Biochem. J. 351, 67-77
• Perou, C.M., et al. (2000). Molecular portraits of human breast tumours. Nature 406, 747-752
• Quigley, C.A., De Bellis, A., Marschke, K.B., el-Awady, M.K., Wilson, E.M., and French, F.S. (1995). Androgen receptor defects: historical, clinical, and molecular perspectives. Endocr. Rev. 16, 271-321
• Rey, R., and Picard, J.-Y. (1998). Embryology and endocrinology of genital development. Baillieres Clin. Endocrinol. Metab. 12, 17-33
• Rodriguez-Esteban, C., Tsukui, T., Yonei, S., Magallon, J., Tamura, K., and Izpisua Belmonte, J.C. (1999). The T-box genes Tbx4 and Tbx5 regulate limb outgrowth and identity. Nature 29, 814-818
• Stillman, S.C., Evans, B.A., and Hughes, I.A. (1991). Androgen dependent stimulation of aromatase activity in genital skin fibroblasts from normals and patients with androgen insensitivity. Clin. Endocrinol. 35, 533-538
• Takeda, H., Mizuno, T., and Lasnitzki, I. (1985). Autoradiographic studies of androgen-binding sites in the rat urogenital sinus and postnatal prostate. J. Endocr. 104, 87-92
• Tobey, R.A., Valdez, J.G., and Crissman, H.A. (1988). Synchronization of human diploid fibroblasts at multiple stages of the cell cycle. Exp. Cell Res. 179, 400-416
• Tusher, V.G., Tibshirani, R., and Chu, G. (2001). Significance analysis of microarrays applied to the ionizing radiation response. Proc. Natl. Acad. Sci. U S A 98, 5116-5121
• Vonesch, J.L., Nakshatri, H., Philippe, M., Chambon, P., & Dolle, P. (1994). Stage and tissue-specific expression of the alcohol dehydrogenase 1 (Adh-1) gene during mouse development. Dev. Dyn. 199, 199-213
• Woods, A. (2001). Syndecans: transmembrane modulators of adhesion and matrix assembly. J. Clin. Invest. 107, 935-941
• Ueda, T., Bruchovsky, N., and Sadar, M.D. (2002). Activation of the androgen receptor N-terminal domain by interleukin-6 via MAPK and STAT3 signal transduction pathways. J. Biol. Chem. 277, 7076-7085
penile foreskin, normal male
labia majora, AIS

HOXA13
WNT2
AKR1C1
FOXP2
MAF
TBX5
BMP4
penile foreskin, normal male
labia majora, AIS, somatic mosaicism
prostate, normal male
gonads, AIS
abdomen / forearm, normal male
labia majora, AIS
ARD842, CAIS, F, GOF, 1 x ETOH, 24h
ARD842, CAIS, F, GOF, 1 x 1000nM DHT, 24h
ARD842, CAIS, F, GOF, 1 x 100nM DHT, 24h
ARD531, CAIS, F, GOF, 6 x 100nM DHT, 14d
ARD531, CAIS, F, GOF, 6 x ETOH, 14d
ARD291, CAIS, F, GOF, 6 x 100nM DHT, 14d
ARD291, CAIS, F, GOF, 6 x ETOH, 14d
ARD842, CAIS, F, GOF, 6 x 100nM DHT, 14d
ARD842, CAIS, F, GOF, 6 x ETOH, 14d
N-58, NORM, M, GSF, 6 x 100nM DHT, 14d
N-58, NORM, M, GSF, 6 x ETOH, 14d
N-33, NORM, M, GSF, 6 x 100nM DHT, 14d
N-33, NORM, M, GSF, 6 x ETOH, 14d
N-56, NORM, M, GSF, 6 x 100nM DHT, 14d
N-56, NORM, M, GSF, 6 x ETOH, 14d
N-56, NORM, M, GSF, 1 x 1000nM DHT, 24h
N-56, NORM, M, GSF, 1 x 100nM DHT, 24h
N-56, NORM, M, GSF, 1 x ETOH, 24h
N-58, NORM, M, GSF, 1 x 100nM DHT, 24h
N-58, NORM, M, GSF, 1 x ETOH, 24h

penile foreskin, normal male
gonads, AIS, female
exponential proliferation
confluence (G0)
Table 1

| cell strain | subtype, origin | external genitalia / phenotype | age at biopsy years; months | androgen receptor |
|-------------|-----------------|-------------------------------|-----------------------------|-------------------|
| N-33        | GSF, foreskin   | normal fertile male           | 51;11                       | normal (K_d 0.08 nM, B_max 26.40 fmol/mg protein) |
| N-40        | GSF, foreskin   | normal male                   | 7;11                        | normal (K_d 0.09 nM, B_max 32.44 fmol/mg protein) |
| N-52        | GSF, foreskin   | normal male                   | 0;3                         | normal (K_d 0.06 nM, B_max 35.36 fmol/mg protein) |
| N-56        | GSF, foreskin   | normal fertile male           | 42;10                       | normal (K_d 0.07 nM, B_max 13.35 fmol/mg protein) |
| N-57        | GSF, foreskin   | normal male                   | 5;11                        | normal (K_d 0.05 nM, B_max 24.13 fmol/mg protein) |
| N-58        | GSF, foreskin   | normal male                   | 5;11                        | normal (K_d 0.10 nM, B_max 30.61 fmol/mg protein) |
| N-59        | GSF, foreskin   | normal male                   | 0;8                         | normal (K_d 0.07 nM, B_max 40.01 fmol/mg protein) |
| N-60        | GSF, foreskin   | normal male                   | 5;4                         | normal (K_d 0.05 nM, B_max 20.08 fmol/mg protein) |
| N-62        | GSF, foreskin   | normal male                   | 2;0                         | normal (K_d 0.08nM, B_max 86.16 fmol/mg protein) |
| N-AKPZF     | PRF, peripheral zone | normal male        | adult                      | not investigated |
| N-ST4       | ASF, abdominal skin | normal male             | 46;0                       | not investigated |
| N-LS12      | FSF, forearm skin | normal male            | 36;0                       | not investigated |
| ARD1097     | GSF, labia majora | normal female          | 1;3                        | CAIS, Pro390Ser + Arg855Gly, negative androgen binding |
| ARD411      | GSF, labia majora | normal female          | 0;4                        | CAIS, Arg855Cys, negative androgen binding |
| ARD682      | GSF, labia majora | normal female          | 14;10                      | CAIS, no mutation in AR-gene, negative androgen binding, no AR-protein in Western immunoblot |
| ARD402      | GSF, labia majora | normal female          | 1;0                        | CAIS, no mutation in AR-gene, negative androgen binding, very low AR-mRNA transcription, no AR-protein in Western immunoblot |
| ARD377      | GSF, labia majora | predominantly female     | 1;2                        | AIS4, Ile841Ser, (K_d 0.55nM; B_max 17.01 fmol/mg protein), post zygotic mutation (somatic mosaicism) |
| ARD842      | GOF, gonad      | normal female          | 38;0                       | CAIS, 26bp deletion exon 1 (141-150), frameshift, premature stop codon, negative androgen binding |
| ARD1004     | GOF, gonad      | normal female          | 17;4                       | CAIS, Val866Met, negative androgen binding |
| ARD291      | GOF, gonad      | normal female          | 18;4                       | CAIS, Phe794Ser, negative androgen binding |
| ARD531      | GOF, gonad      | normal female          | 35;2                       | CAIS, Ala765Thr, negative androgen binding |
| ARD557      | GOF, gonad      | normal female          | 6;6                        | CAIS, donor splice site exon 2 / intron 2, negative androgen binding |
| ARD465      | GSF, labia majora | normal female        | 5;5                        | CAIS, Glu287stop, low expression of wild type AR (K_d 0.11nM; B_max 3.6 fmol/mg protein), post zygotic mutation (somatic mosaicism) |
| ARD364      | GSF, labia majora | predominantly female   | 23;0                       | AIS4, Leu 172 stop, high expression of wild type AR (K_d 0.06 nM, B_max 22.6 fmol/mg protein), post zygotic mutation (somatic mosaicism) (6) |