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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

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Abstract

Background: Androgen insensitivity syndrome (AIS) comprises a range of phenotypes from male infertility to complete feminization. Most individuals with AIS carry germline mutations of the androgen receptor (AR) that interfere with or ablate its function. As genital fibroblasts retain expression of the AR *in vitro*, we used genital skin fibroblasts from normal males and 46,XY females with complete AIS due to known *AR* mutations to gain insights into the role of the AR in human genital differentiation.

Results: Using DNA microarrays representing 32,968 different genes, we identified 404 transcripts with significant differences in transcription levels 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. On the basis of animal studies and human genetic syndromes, several of these genes are known to have specific roles in genital differentiation. Remarkably, genital fibroblasts from both normal and AIS-affected individuals showed no transcriptional response to dihydrotestosterone treatment despite expression of the AR.

Conclusions: The results suggest that in addition to differences in the anatomic origin of the cells, androgen signaling during prenatal development contributes to setting long-lasting, androgen-independent transcriptional programs in genital fibroblasts. Our findings have broad implications in understanding the establishment and the stability of sexual dimorphism in human genital development.

Background

Development of the male genitalia is largely controlled by cells in the urogenital mesenchyme that express androgen receptors (AR) [1,2]. Germline 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 levels of serum testosterone [3–5].

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 mesenchyme-derived stromal cells from normal males to those from individuals affected with AIS. As cultured genital fibroblasts originate from the 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.

Results

Baseline gene transcription 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 germline inactivation of the AR as a result of mutation. We could not identify mutations in the *AR* gene in two phenotypically female individuals 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 nine normal males and from the labia majora of five AIS-affected, 46,XY individuals with female external genitalia. The AR status of all genital 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 (G_0) 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 (Figure 1). We then identified 404 unique transcripts (represented by 487 total cDNAs) with significant differences in expression levels between normal and

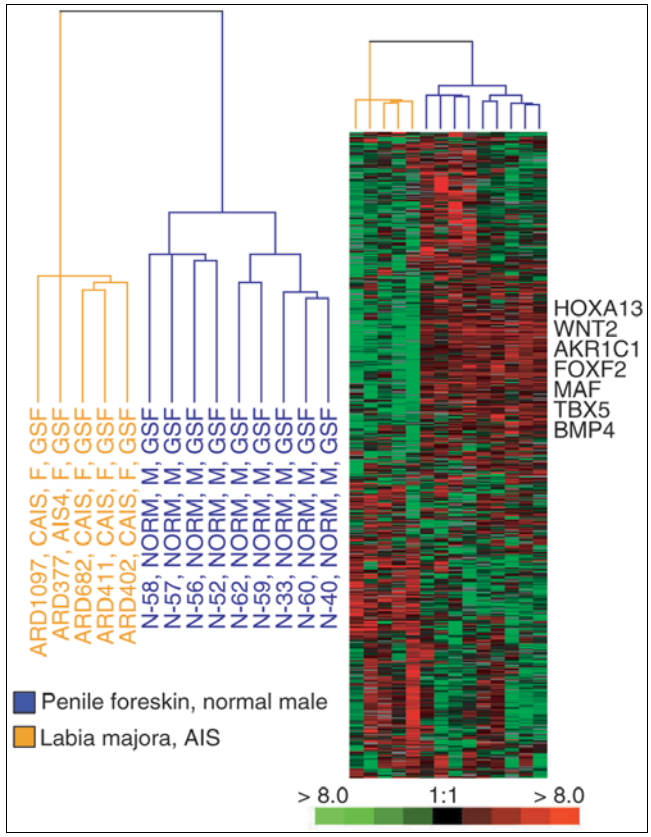

Figure 1

Unsupervised hierarchical cluster analysis of genes and experiments of nine normal genital skin fibroblast lines (penile foreskin) and five AIS genital skin fibroblast lines (labia majora). Only transcripts whose $\log_2$ red/green ratio differed from the mean expression level across all experiments by at least 1.1 in at least four experiments are displayed (620 cDNAs). The dendrogram of the array experiments (above and repeated on the left) reflects the similarity of the samples with respect to their gene-expression patterns. F, female external genitalia; M, normal male external genitalia; NORM, normal male control; AIS4, AIS with predominantly female phenotype (slight enlargement of the clitoris); CAIS, complete androgen-insensitivity syndrome; GSF, genital skin fibroblast. 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 scale ranges from -8 to +8 in $\log_2$ space.

AIS genital fibroblasts using the significance analysis of microarrays (SAM)-procedure [6], with a false discovery rate of less than 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 (Figure 2). In addition to the 14 genital-skin fibroblast lines used to generate the SAM list, we included five 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, forearm fibroblasts

Table 1

Cell strains

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 0.08 nM, 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 0.55 nM; B_{max} 17.01 fmol/mg protein)
ARD842	GOF, gonad	Normal female	38;0	CAIS, 26 bp 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 amdrogen 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 0.11 nM; 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)

ARD, strain-ID; ASF, abdominal skin fibroblast; FSF, forearm skin fibroblast; GOF, gonadal fibroblast; GSF, genital skin fibroblast; PRF, prostate fibroblast. Normal ranges for androgen (methyltrienolone) binding: B_{max} (binding capacity): 13.35-115.98 fmol/mg protein; K_d (dissociation constant): 0.03-0.13 nM.

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 [7]. 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 (Figure 2). The latter group contained all five 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 ARD465 showed gene-expression patterns that most resembled normal male foreskin (Figure 2).

Comparison of expression patterns in genital 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 (HOXA13) and T-box proteins (TBX) 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

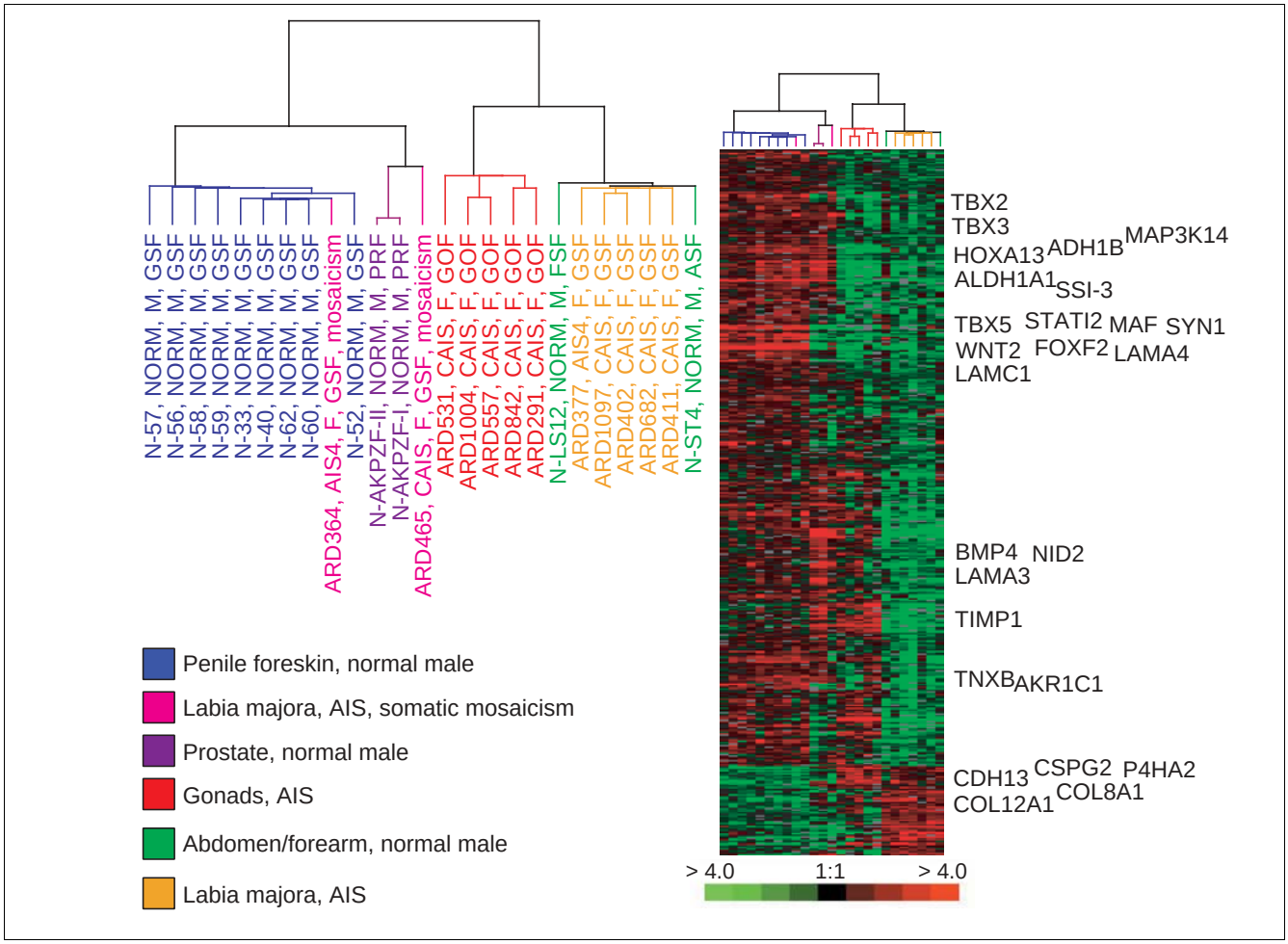

Figure 2

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. GSF, genital skin fibroblast; PRF, prostate fibroblast; GOF, gonadal fibroblast; FSF, forearm skin fibroblast; ASF, abdominal skin fibroblast; other abbreviations are as in Figure 1 and Table 1. The scale ranges from -4 to +4 in $\log_2$ space. For the complete dataset, see Additional data files and [36].

fibroblasts and at low levels in all AIS and extragenital fibroblasts (Figure 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 (Figure 2). *TBX2* showed an almost identical expression profile to *TBX3*, whereas high *TBX5* expression appeared to be restricted to foreskin fibroblasts from normal males (including those from the phenotypically female mosaic patient ARD364). *BMP4* (bone morphogenetic protein 4) was predominantly expressed in foreskin fibroblasts from normal males and in prostate fibroblasts (Figure 2). *WNT2* (wingless-type MMTV integration site family member 2) was part of a small gene cluster with high expression in normal male foreskin fibroblasts that distinguished these samples from all other fibroblasts (Figure 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* (encoding 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*) and tissue inhibitor of metalloproteinase 1 (*TIMP1*) all showed relatively high expression levels compared to AIS-derived fibroblasts (Figure 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 (Figure 2). Other differentially expressed genes, such as mitogen-activated protein kinase 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 dihydrotestosterone (DHT), under culture conditions similar to those that were reported to produce aromatase induction in these cells [8]. Cells were treated with DHT (100-1,000 nM) both at confluency (G_0) and during exponential growth, and transcript levels were assessed using DNA microarrays. Unsupervised hierarchical clustering did not disclose any obvious differences in gene-expression patterns between DHT-treated and ethanol-treated fibroblasts, either in normal controls or in AIS-derived cell lines. We treated LNCaP prostate cancer cells with androgen under similar conditions and readily identified nearly 500 transcripts modulated by androgen with unsupervised hierarchical clustering analysis and with supervised methods ([9] and data not shown). 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. Additional experiments using physiological concentrations of androgen (for example, 0.01 - 1 nM methyltrienolone) also failed to disclose any androgen-responsive genes (data not shown). In contrast, SAM analysis identified 1,664 transcripts that differed significantly between proliferating and confluent cells, and 1,232 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 (Figure 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* (Figure 3).

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 that 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 morphogenetic 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 [10]. The differences in gene expression we observed in AIS fibroblasts of gonadal origin compared to those of labial origin support this view (Figure 3). We have observed consistent and characteristic differences in the gene-expression patterns of skin fibroblasts derived from different locations on the body [11]. However, the current set of experiments suggests that the androgen receptor has a cell-autonomous role which contributes to 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 relevant determinant of baseline gene expression in genital skin fibroblasts. Both 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 [12]. ARD364, which showed AR protein expression and binding in the range of normal male foreskin fibroblasts ([7] and see Table 1), despite its origin from anatomically female genitalia, had a gene-expression pattern indistinguishable from foreskin fibroblasts of normal males (Figure 2). The second fibroblast line from an AR mosaic patient, ARD465 (Table 1), had very low 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 (Figure 2).

The discrepancy between the female phenotype of these mosaic individuals despite expression of the wild-type AR in cultured genital skin fibroblasts is not resolved to date [7]. It may be explained by a time-dependent rise of an originally small fraction of cells containing the wild-type *AR* allele in the mosaic genital mesenchyme during prenatal and postnatal development, or by differences between *in vivo* and *in vitro* conditions. Yet, the documented expression of the wild-type AR in cultures of these labial cells supports the idea that the AR status of the fibroblast was an important intrinsic determinant of the basal transcription patterns we identified. Therefore, the AR appears to be involved in setting long-lasting gene-expression patterns in genital skin fibroblasts.

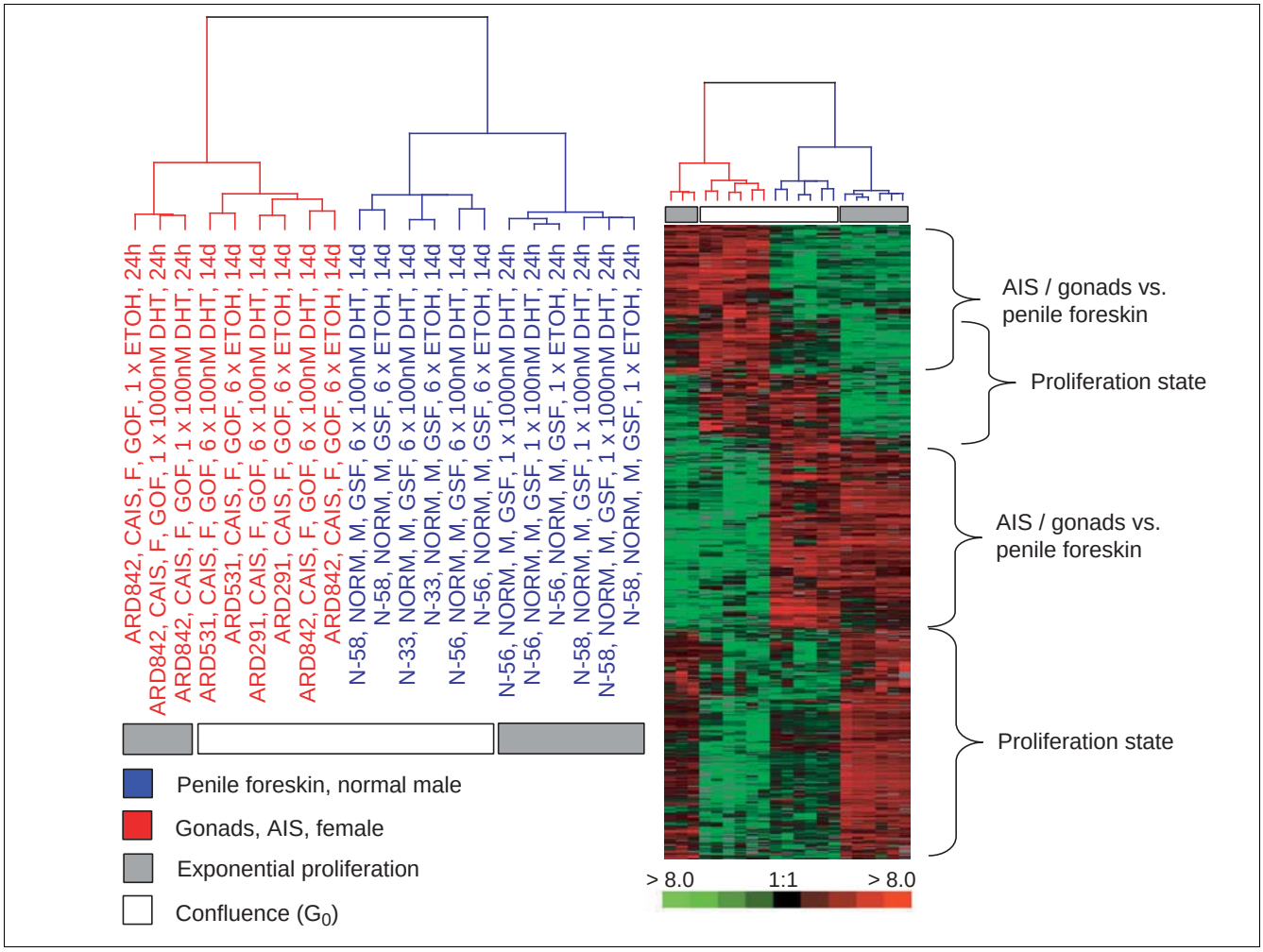

Figure 3
Hierarchical cluster analysis of genes and experiments with different DHT-treatment regimens. Shown are 686 transcripts whose $\log_2$ red/green ratio differed from the mean expression level across all experiments by at least 1.5 in at least three experiments. The analysis is based on 2,862 transcripts identified by SAM analysis that distinguish between normal genital skin fibroblasts and gonadal fibroblasts from 46,XY female AIS patients, and between proliferating and confluent fibroblasts. The color code in the dendrogram depicts the origin of the fibroblast cultures. The gray and white bars at the top of the cluster indicate the proliferation state of the samples. On the right, the regions of the cluster diagram that differentiate between normal and AIS-derived fibroblasts, and proliferating and confluent cells, respectively, are indicated. No differences in transcript levels could be discerned between DHT-treated and control cells in either normal foreskin fibroblasts or fibroblasts from AIS-affected 46,XY females. Abbreviations are as in Figure 2 and Table 1. The scale ranges from -8 to +8 in $\log_2$ space. For the dataset of 686 genes used for the cluster analysis and the complete dataset of 2,862 genes, see Additional data files and [36].

Comparison of gene-expression patterns in genital fibroblasts from normal and AIS-affected individuals, and in 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 [13–15]. The differential expression of proteoglycans, collagens and cell adhesion molecules (for example 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 [16] 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 [17]. 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 MAP kinase and STAT pathways are involved in AR-dependent regulation and in ligand-independent activation of the AR [18]. 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 [19,20].

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 *DWnt2*. Mutations in *HOXA13* can cause distal limb and urogenital-tract malformations such as male hypospadias in hand-foot-genital syndrome [21]. T-box genes (*TBX*) are essential early regulators of limb development and also appear to be involved in male genital development [22,23]. Mutations in *TBX3* cause the ulnar-mammary syndrome characterized by limb, apocrine, and genital developmental abnormalities [23]. Expression of T-box genes 2, 3, and 5 was significantly higher in normal male foreskin fibroblasts than in AIS genital fibroblasts. *BMP4* has been implicated in ductal budding and branching during prostate development [24] and a potential role of BMP4 in external genital development has also been postulated [25]. *DWnt2* has been found to have roles in sex-specific cell determination in the gonads and genital disc of *Drosophila* [26]. 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.

As normal genital skin fibroblasts of 46,XY male individuals express the AR *in vitro* (see Table 1 and [4,7]), 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. We have previously used a similar approach to delineate the transcriptional programs activated in prostate cancer cells in response to androgen [9]. We had hoped that comparison of transcriptional responses of normal fibroblasts to those from AIS-affected individuals with varying degrees of genital ambiguity would provide still further insights into androgen's role in genital morphogenesis. However, we were unable to detect any significant changes in gene-expression patterns in cultured, AR-expressing genital fibroblasts or in AIS-derived fibroblasts in response to androgens. Although two previous reports have shown increases in aromatase enzymatic activity in genital skin fibroblasts treated with dihydrotestosterone (DHT) [8,27], others have failed to observe changes in aromatase activity in response to androgen [28]. In agreement with our findings, Elmlinger *et al.* found significantly different baseline expression levels of insulin-like growth factor (IGF) and insulin-like growth factor binding protein (IGFBP) between normal and AIS-derived genital skin fibroblasts, and could not detect changes in transcript levels in response to androgen treatment [29]. In normal genital fibroblasts, androgen-responsive reporter genes can only be activated by expression of co-transfected AR in the presence of ligand [30]. Therefore, endogenous AR expression itself may be insufficient in genital skin fibroblasts to elicit a transcriptional response. Moreover, the lack of detectable changes in transcript levels for any of the 30,000 genes in the AIS-fibroblasts virtually excludes the possibility that DHT or R1881 could be acting through other steroid receptors or other signaling pathways.

The differences in androgen responsiveness we have observed between normal genital fibroblasts and prostate cancer epithelial cells *in vitro* might reflect the responses seen *in vivo*. 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 or no change in the external genitalia. 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. In mice, stromal androgen responsiveness is restricted to the earliest stages in prostate development, and later the epithelial compartment becomes responsive and remains so [1]. This responsiveness may be mediated through the expression of specific AR co-regulators. Compared to LNCaP cells, normal male genital fibroblasts show distinctly lower baseline expression of several AR co-regulators (such as NCOA2 (GRIP-1), NCOA3 (TRAM-1), ARA54 (RNF14), data not shown). Thus, genital fibroblasts may express critical AR co-regulators at discrete times during development that allow them to respond by setting up long-lasting transcriptional programs that underlie the genesis and maintenance of genital morphology.

Conclusions

Our data suggest 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. For further discrimination of androgen-independent positional effects from prenatal androgen actions on expression phenotypes of genital fibroblast strains, comparative expression profiling of homologous genital tissues is needed. 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 of genital development *in vivo* in the presence and absence of androgen may provide further insights into the role of androgen in genital development.

Materials and methods

The study was approved by the ethical committee of the 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 (Department of Biochemistry, Stanford University). Peripheral zone prostate fibroblasts were a gift from D. Peehl (Department 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 using methyltrienolone (R1881, 17β-hydroxy-17α-methyl-4,9,11-estrotrien-3-one) and androgen receptor sequencing have been described previously [7].

Cell culture and hormone treatment

For determination of basal gene-expression profiles without androgen stimulation, fibroblasts were cultured on 150-mm plastic dishes at 37°C with 5% CO_2. To eliminate possible artifacts due to differing states of proliferation, cells were grown to confluence, at which point they enter G_0 arrest [31]. They were maintained in phenol-red-free DMEM F12 (Dulbecco's modified Eagle Medium with the nutrient mix F12; Gibco) containing L-glutamine, 15 mM 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 1 N NaOH and the medium was exchanged every 48 h. At day 13 the last media exchange was carried out and 96 h 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 [8,27]. In the first, cells were grown to confluence as described above using phenol-red-free DMEM F12 containing L-glutamine, 15 mM 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 every 48 h, cells received either ethanol in a final dilution of 1:100,000 or 100 nM dihydrotestosterone (DHT) dissolved in ethanol. The last DHT treatment was administered with the last media exchange 96 h before lysate preparation. In total, six doses of either ethanol or 100 nM DHT were given.

In the second set of experiments, cells were cultured to confluence for 14 days as described above. They were then trypsinized and seeded at a density of 3,000 cells per cm^2 in 150-mm plates. Twenty-four hours later, medium was removed, and cells were washed three times with new media containing 12.9% D/S-FCS, then cultured for another 24-h interval in the absence of androgens. Cells were then treated with either 1:100,000 ethanol, 100 nM or 1,000 nM DHT dissolved in ethanol. After 24 h incubation, exponentially growing cells were harvested. LNCaP cells, passaged and treated under similar conditions, were used as a positive control for androgen reponsiveness.

RNA isolation and cDNA labeling

Protocols for mRNA preparation and cDNA labeling are available online [32]. mRNA (2 μg) 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 that we have described previously [33].

Microarrays and hybridizations

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

Microarray data analysis

Only spots with fluorescence signals 1.5-fold greater than 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 $\log_2$ (ratio) of 0. We restricted our analysis to genes with measurable expression in 80% of the samples we analyzed. We used the SAM procedure [6] to identify genes with statistically significant differences in baseline expression levels between normal and AIS genital fibroblasts. The SAM procedure computes a two-sample T-statistic (for example 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. Gene-expression data were clustered [34,35] and results were visualized using TreeView software [35].

To identify the effects of androgen treatment on gene expression in genital fibroblasts, we carried out a set of 21 DNA microarray analyses of mRNA from normal and AIS genital

fibroblasts. This dataset 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 with reference to the origin of the fibroblast lines, whether the cells were confluent or proliferating, and whether they had been treated with androgen.

Additional data files

The following files are available with the online version of this paper: a figure (Additional data file 1) showing the complete dataset for Figure 1, with associated array tree correlations (atr), complete data table (cdt) and gene tree correlations (gtr) files (Additional data files 2, 3 and 4); a figure (Additional data file 5) showing the complete dataset for Figure 2, with associated atr, cdt and gtr files (Additional data files 6, 7 and 8); a figure (Additional data file 9) showing the complete dataset for Figure 3, with associated atr, cdt and gtr files (Additional data files 10, 11 and 12). Figure 3 contains 686 transcripts whose $\log_2$ red/green ratio differed from the mean expression level across all experiments by at least 1.5 in at least three experiments of the treatment series. The analysis was based on 2,862 transcripts that differed significantly between proliferating and confluent cells and between fibroblasts derived from AIS-affected individuals and normal male foreskin, respectively, as identified by SAM analysis of the treatment series. The complete 2,862 genes are displayed in two further figures (Additional data files 13 and 14) with associated atr, cdt and gtr files (Additional data files 15, 16 and 17). All files are also available online at [36].

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