SOS1 OVER-EXPRESSION IN GENITAL SKIN FIBROBLASTS FROM HIRSUTE WOMEN: A PUTATIVE ROLE OF THE SOS1/RAS PATHWAY IN THE PATHOGENESIS OF HIRSUTISM

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Hirsutism is the development of androgen-dependent terminal body hair in women in places in which terminal hair are normally not found. It is often associated with hyperandrogenemia and/or polycystic ovary syndrome (PCOS), but the existence of uncommom hirsutism forms that are not related with altered androgen plasma levels lead also to the definition of "idiopathic hirsutism". Although the pathophysiology of hirsutism has been linked to increasing 5-alpha reductase (*SRD5A*) activity and to an alteration of the androgen receptor (*AR*) transcriptional machinery, many aspects remain unclear. In particular the relationships between androgens and local factors are poorly understood. In the present paper, we selected for a genital skin biopsy, 8 women affected with severe hirsutism (Ferriman&Gallway score >25) but with normal plasma androgen levels with the exception of slight higher serum 3α-diol-glucuronide levels, and 6 healthy controls and analyzed their androgen- and insulin-specific transcriptional profile using a specific custom low density microarray (AndroChip 2, GPL9164). We identified the over-expression of the Son of Sevenless-1 (*SOS1*) gene in all of the hirsute skin fibroblast primary cell cultures comparing with control healthy women. Since SOS1 is a guanine nucleotide exchange factor that couples receptor tyrosine

kinases to the RAS signaling pathway that controls cell proliferation and differentiation, we further analyzed SOS1 expression, protein level and RAS signaling activation pathway in an *in vitro* model (NHDF, normal human dermal fibroblasts cell line). NHDF treated for 24h with different concentration of DHT and T showed an increase in SOS1 levels (both mRNA and protein) and also an activation of the RAS pathway.

Our *in vivo* and *in vitro* data represent a novel preliminary observation that factors activating SOS1 could act as local proliferative modulators linked to the androgen pathway in the pilosebaceous unit. SOS1 overexpression may play a role in the regulation of the RAS/mitogen activated protein kinase pathway in the skin, in the hair follicle proliferation and cell cycle, suggesting new perspectives in understanding the pathogenesis of idiopathic hirsutism.

INTRODUCTION

Hirsutism is defined as the presence in women of terminal hairs growing in androgen-dependent areas affecting 5-10% of women in the reproductive age (1-3). The presence of hirsutism is extremely distressing to patients, with significant negative impact on their psychosocial development (4-5). In the majority of patients, hirsutism is linked to a hyperandrogenism phenotype such as polycystic ovary syndrome (PCOS), androgen secreting tumors, non-classical adrenal hyperplasia (NCAH), Cushing syndrome and syndromes of severe insulin resistance (6-8). However, many women develop idiopathic hirsutism (IH), which occurs in the absence of plasma hyperandrogenemia, ovary ultrasound abnormalities, and abnormal ovulatory function.

Clinical diagnosis of hirsutism is a relatively subjective process based on visual assessment of hair type and hair growth. The most common method used to grade hair growth is the modified scale of Ferriman-Gallwey (1). This method grades terminal hair growth density at 11 different body sites, using a grading range from 0 (absence of terminal hairs) to 4 (extensive terminal hair growth) for each area. The total score is then correlated with the severity of the problem.

Hair growth in subjects with normal circulating androgens appears to be the result of altered sensitivity of the hair follicle to plasma androgen that is caused by increased availability of 5-alpha-reductase (*SRD5A*) activity in the local environment (9) and/or by modifications of androgen receptor (*AR*) function related to polymorphisms in the *AR* gene (10,11). However, the basis for the variability in the androgen response pattern of the pilosebaceous unit (PSU) is unclear, given that the severity of hirsutism does not always correlate with plasma androgen levels, which vary considerably within and among affected individuals (12, 13). The inequality in the response of PSU to androgens is determined by the interaction between androgen levels and intrinsic assets of PSU rising from differences in genetic programming or in sensitivity to androgens. In this context, idiopathic hirsutism (IH), also called as "hirsutism of unknown cause", has been defined as the condition under which hirsutism occurs even though circulating androgen levels are within the normal range (7). This finding supports the notion that dysregulation of local factors that act as intermediaries in the androgen response, may contribute to the proliferation of hair follicle cells that lead to hirsutism.

We demonstrated that SOS1, a protein involved in the IGF-I/insulin signaling, is over-expressed both *in vivo* (genital skin fibroblast of hirsute women) than in an *in vitro* model of androgen excess syndrome (normal human dermal fibroblast, NHDF cell lines), suggesting that idiopathic hirsutism may be the result of dysregulation of the Ras/mitogen activated protein kinase (MAPK) pathway in the skin.

MATERIALS AND METHODS

Patient selection and skin biopsy

Eight severely hirsute women aged 27.7±2.4 yrs, and six healthy women of similar age were selected from a large cohort of women all presenting with androgen excess syndrome attending for clinical survey the Section of Reproductive Endocrinology of the Unit of Endocrinology at the Fatebenefratelli Hospital in Isola Tiberina, Rome, Italy. They were evaluated for BMI (body mass index), menstrual cycle, serum androgen (including total testosterone), sex hormone binding globulin (SHBG), 3α-diol-glucuronide, DHEA, DHEAS, 17-αOH progesterone, and fasting glucose/insulin ratio (HOMA index) (Tab. 1). The degree of hirsutism in the patient cohort was assessed using the modified Ferriman-Gallway scoring system (1) with a score of ≥10 indicating the presence of hirsutism. Blood for assaying hormones was collected during the follicular phase of the menstrual cycle and in the absence of any hormonal medication. Women who presented at least one of the following criteria: evidence for androgen-secreting tumors, acanthosis nigricans, hyperinsulinemia, Cushing's syndrome, hyperprolactinemia, thyroid dysfunction, and/or a family history of diabetes mellitus, were excluded from this study. The patients selected showed features that could be classified as severely idiopathic hirsute (Ferriman&Gallway index score > 25) compared to female of similar age and BMI used as controls, because they all showed regular menstrual cycles, no hyperandrogenemia, and no ultrasonographic evidence of PCOS. We perfomed a genital skin biopsy to the patients and control women at the level of the lower abdomen, an area where androgen receptors are densely expressed, during the follicular phase of the menstrual cycle (7°-8°GC), using a 4 mm diameter *biopsy punch* (Stiefel®).

This study was performed according to the principles of the Helsinki Declaration. Both patients and controls were provided with written informed consent for the collection of samples and subsequent genetic analysis. The biopsies were performed under institutionally approved protocols (IRBs for Tor Vergata University, and the Fatebenefratelli Hospital, Rome, Italy). Anonymous codes were assigned to all samples.

Genital skin primary fibroblast and NHDF cell cultures

Primary fibroblast cultures were established from the genital skin biopsies obtained by the eight hirsute patients and the six healthy women, by dicing the sample into small pieces with a scalpel (14). Cultures were maintained in Dulbecco's modified Eagle's-F12 medium (DME-F12, without phenol red) (EuroClone, Italy) supplemented with 10% fetal bovine serum (FBS) (EuroClone, Italy), 1% L-glutamine and 1% antibiotics. After three subcultures, cells were harvested and RNA was isolated.

Adult normal human dermal fibroblasts (NHDF) were purchased from Lonza (Walkersville, MD). Cells were propagated and maintained in fibroblast medium added with human fibroblast growth factor B (hFGF-B), insulin, fetal bovine serum and gentamicin/amphotericin-B as indicated by manufacturer's instructions, at 37°C in a humidified atmosphere of 5% CO_2. When the cultures reached 75–85% confluency, cells were starved in a culture medium not supplemented for 12h and then treated with Testosterone (T) (Sigma, USA), Dihidrotestosterone (DHT) (Sigma, USA), bicalutamide

(BIC) (AstraZeneca, Basiglio, Italy) or vehicle (ethanol). NHDF cells were treated with different concentration of T (1nM, 10nM and 100nM), DHT (0.1nM, 1nM and 10nM) and BIC for 3h, 6h and 24h. After each treatment cell extracts were collected for total RNA and protein isolation used for qRT-PCR, Western Blot and RAS activity assay.

RNA extraction

Cells (both genital skin fibroblast primary cell and NHDF) were washed twice with 1X Phosphate Buffer Saline (PBS, Invitrogen, Carlsbad, USA), collected with scraper and immediately lysated in 1 ml of TRIzol^{TM} Reagent (Invitrogen, Carlsbad, USA). Total RNA was isolated according to the manufacturer's instructions. Aliquots of RNA were then quantified and assessed for quality by spectrophotometry (Nanodrop, Wilmington, USA) and agarose gel electrophoresis.

Microarray analysis

We used a pathway specific low-density custom oligoarray, "AndroChip-2" (Gene Expression Omnibus database, http://www.ncbi.nlm.nih.gov/geo/, GPL9164) as described in *Minella et al 2009*) (15). The microarray experiment was replicated in duplicate and the raw experimental data were submitted to the GEO database with the accession number GSE18022. Inter-individual variation and other confounding events (16), were minimized by separating patients and controls into two distinct pools of total RNA that were then used for the microarray experiments. Briefly, first strand cDNA was synthesized with the Superscript Indirect cDNA-labeling system following the relevant protocol (Invitrogen, Carlsbad, USA) starting from 10 μg of total RNA of each pool (hirsute and control one) as described in

Minella et al 2010 (15). Images were analyzed with GenePix Pro5.0 software (Axon instruments, Union City). The processing data were made as described in *Minella et al 2010* (15).

The significance of the observed modulation of each gene was determined using Student's *t*-test with the Welsh correction controlling for the multiplicity of testing. Only genes with a satisfactory absolute FC at least $\geq \pm 1.5$ and statistically significant (*p* value <0.05) were considered.

qRT-PCR

The microarray data were validated on selected genes by quantitative real-time PCR (qRT-PCR) (17). Total RNA was reverse-transcribed to cDNA with the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, USA) following the relevant protocol. qRT-PCR was performed using the Taqman system, and the expression levels of six genes *SOS1* (Hs00893133_m1), *AR* (Hs00171172_m1), *SRD5A2* (Hs00165843_m1); *FN1* (Hs01549940_m1), VCAM1 (Hs01003372_m1), *CASP9* (Hs00609647_m1) and the *β-actin* internal reference were determined by multiplex PCR with the Assay-on-Demand^{TM} gene expression reagents labeled with 6 carboxyfluorescein FAM or VIC (Applied Biosystems, Foster City, USA). All PCR amplifications were performed using the Taqman Universal PCR Master Mix and ABI PRISM7000 Sequence Detection System, and all qRT-PCRs were carried out in triplicate and repeated at least twice. The comparative threshold (CT) cycle was used to determine the gene expression level relative to the calibrator RNA from the controls. Steady state mRNA levels were expressed relative to the calibrator as "n-fold" differences. The CT value for

each gene was normalized using the formula $\Delta CT = Ct(gene) - Ct(\beta\text{-}actin)$. The relative gene expression levels were determined according to the following formula: $\Delta\Delta CT = \Delta CT_{sample} - \Delta CT_{calibrator}$.

The value used to plot relative gene expression was determined using the expression Fold Change (FC)$=2^{-\Delta\Delta CT}$ (18).

Western blot analysis

Genital skin fibroblasts from hirsute patients and healthy controls were harvested by scraping into PBS and pelleting at 200xg for 10 min. The pellet was homogenized by sonication (Ultra-turrax-T8; IKA-WERKE, GmbH & Co. KG, Germany) in RIPA buffer containing a protease inhibitor cocktail (Pierce Chemical Co., Rockford, IL).

Total protein was determined by the Bradford method (19) using BSA as a standard. Equal amounts of protein (80 μg) from each lysate were loaded onto an 8% w/v SDS-polyacrylamide gel for electrophoretic separation. The proteins were blotted onto a nitrocellulose membrane (0.45 μm; Hybond-P Amersham Biosciences, UK). SOS1 protein was detected by probing with a rabbit polyclonal primary antibody (Santa Cruz Biotechnology, CA, USA) diluted 1:400. Blots were incubated with a peroxidase-conjugated AffiniPure Goat Anti-Mouse IgG (H+L) secondary antibody (Jackson ImmunoResearch, UK) diluted 1:3000. Proteins were detected by chemiluminescence (ECL Plus Detection Reagents, Amersham Biosciences, UK).

Densitometric analysis was performed using Optiquant Acquisition & Analysis software 03.10 (Packard Instrument & Co.), and the total amount of SOS1 protein for each hirsute patient and each control and for the treated and untreated NHDF cells, were quantified in the same gel using β-actin as a housekeeping control and presented as a bar chart for each sample as the mean ± standard deviation (SD).

RAS activity assay

After 24h of androgens treatment, whole NDHF cellular extract were collected as indicated by Ras GTPase chemi ELISA kit (Active Motif, Belgium) instruction manual. Proteins concentrations were determined using Bradford assay (19) (Biorad, Segrate MI, Italy). Ras activity was measured using a Ras GTPase chemi ELISA kit (Active Motif, Belgium), following manufacturer's instruction. 100μg of whole cellular extract per well was used; each sample was tested in duplicate. Chemiluminescence was detected using luminometer (Victor[3]V, Perkin Elmer, Massachusetts, USA).

Sequence analysis of the *SOS1* promoter and 3' untranslated region (3'UTR)

Genomic DNA was extracted from peripheral blood of the eight hirsute patients that showed enhanced *SOS1* expression relative to the controls with the Biorobot EZ1 automated system and the EZ1 DNA Blood Kit (Qiagen, Milan, Italy). We sequenced both a 1,272 bp promoter region upstream of exon 1 (for the primer see Supplementary Table A) than a 4,573 bp 3'UTR region downstream of the TGA stop codon up (for the primer see Supplementary Table B). Primers were designed with the Primer3 program to give products in the range of 300-600 bp. PCR amplifications were performed using 200 ng of DNA following the standard AmpliTaq Gold DNA-Polymerase protocol (Applied Biosystems, Foster City, USA). Amplicons were purified enzymatically

with 20000 U/ml ExonucleaseI and 10000 U/ml CIP alkaline phosphatase and directly sequenced with a BigDye-Terminator-v 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, USA). The sequencing reaction products were purified using CentriSep columns (Princeton Separations, Adelphia, USA). Electrophoretic separation was carried out on an ABI Prism3130xL Genetic Analyzer (Applied Biosystems, Foster City, USA). The sequences were aligned and compared to the revised Cambridge Reference Sequence (rCRS) using the sequence analysis and alignment software SeqScape (Version2.0, Applied Biosystems).

Statistical Analysis

Means and standard deviations (SD) were calculated for the case and control groups at mRNA and protein expression levels, and the statistical significances were determined with the non-parametric Mann-Whitney U-test through the Wilcox-test function of the R statistical software.

Furthermore, we quantified SOS1 protein over-production by WB in each hirsute patient (obtained from densitometric analysis) and correlated it with the patient's degree of hirsutism as indicated by their Ferriman&Gallwey index score. For this purpose we performed a Pearson's correlation, which reflects the degree of the linear relationship between two variables (percentage of SOS1 protein over-production and Ferriman&Gallwey index score). The relationship between SOS1 protein overproduction and the degree of hirsutism in each hirsute patient is depicted in the scatterplot shown in Figure II D.

For the RAS activity assay, statistical analysis of results was performed by a two-way factorial analysis of variance (ANOVA) and a one-way ANOVA followed by a Bonferroni's test. P values lower than 0.05 were considered evidence for statistical significance.

RESULTS

Clinical and hormone profile of selected women

For the genital skin biopsy, we selected 8 severely hirsute women on the basis of mean Ferriman-Gallwey score of 25±2.8. The hormonal profiles of all subjects are presented in Tab. 1. Both the hirsute patients and the controls had normal testosterone (<80 ng/dL), androstenedione (<2.8 ng/dL), and DHEAS (<1400 µg/ml). The hirsute women had slight but significantly (p value <0.05) higher serum levels of an end-product in the pathway of skin androgen metabolism (3α-diol-glucuronide) than the controls. The fasting serum insulin and glucose levels as well as the HOMA-index were less than 2.6 in the two groups, indicating a lack of reduced insulin sensitivity.

	Ref. range	Hirsute patients F&G>20 n=8	Controls F&G=0-5 n=6
FSH (mIU/ml)	2.5-13	5.4±0.4	4.8±0.2
LH (mIU/ml)	2.4-12.6	6.2±0.3	5.7±0.5
Estradiol (pg/ml)	25-195	72.1±7.8	82.4±6.7
Delta4AD (ng/ml)	1-2.5	1.8±0.4	2.2±0.4
Testosterone (ng/dL)	10-80	63.3±11.6	48.0±10.6
SHBG (nmol/L)	20-90	28.9±2.8	41.5±3.8
DHEAS (µg/dl)	800-1400	870.8±84.7	960.3±74.7
3αdiolo glucuronide (ng/ml)	0.2-5.5	3.7±1.4	0.8±0.3
Fasting glucose (mg/dl)	60-105	81.5±4.7	78.8±2.5
Fasting insuline (mU/ml)	3-17	6.4±1.3	6.7±0.8
HOMA index	< 2.6	1.9±0.4	1.4±0.3

Tab.1 Androgen and insulin profiles of the hirsute patients selected for genital skin biopsy and control women: a biochemical characterization.

Microarray and qRT-PCR gene expression analysis: identification of *SOS1* gene over-expression in hirsute patients

We assessed the expression profiles of 190 genes involved in androgen metabolism as well as genes of the IGF1/insulin signaling pathway (AndroChip 2) expected to have a role in the hirsutism pathogenesis by microarray. The microarray experiments identified a total of four genes that were differentially expressed (absolute FC>±1.5; p <0.05) in hirsute patient fibroblasts. Two of these genes, *SOS1* (*Son of Sevenless-1;* FC= +1.6 and p value= 5.43e-0.5) and *CASP9* (*Caspase 9;* FC= +1.5 and p value= 0.00096), were over-expressed and two genes, *FN1* (*Fibronectin1;* FC= -1.5 and p value=0.00116) and *VCAM1* (*Vascular Cell Adhesion 1;* FC= -1.5 and p value= 0.0026), were under-expressed.
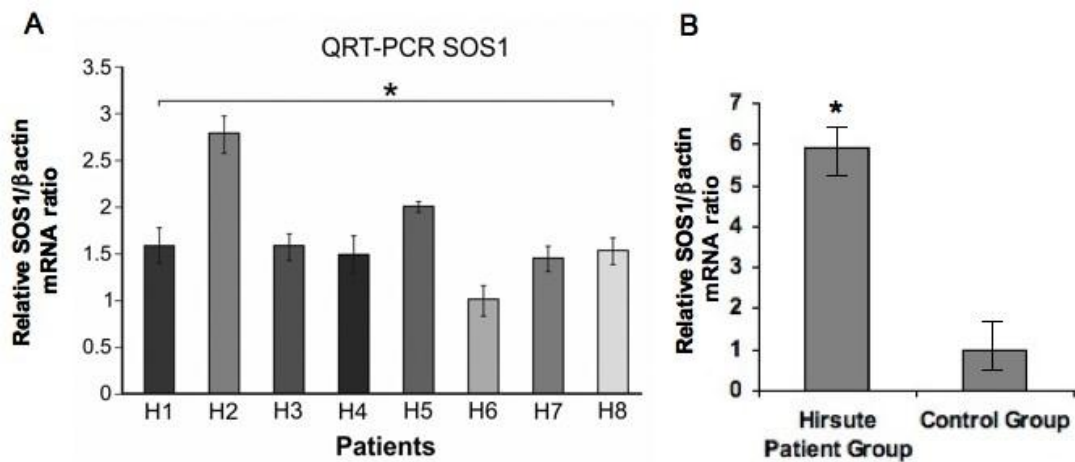
Fig.I *SOS1* **gene over-expression analyzed by qRT-PCR in the hirsute patients and relative controls.**

A) *SOS1* over-expression for each hirsute patient is indicated as Relative *SOS1/β-actin* mRNA ratio calculated as described in the Materials and Methods section. Error bars represent the relative standard deviation. The * indicated a statistically significance p value < 0.05.

B) The bar chart represents the mRNA expression levels of the hirsute patient group (n=8) and the control group (n=6) as the mean of the Relative *SOS1/ β-actin* mRNA ratio obtained from the hirsute patient. The * indicated a statistically significance p value < 0.05 difference between the hirsute patients group and the controls one.

All the differentially expressed genes identified by the AndroChip 2 microarray were re-assessed by qRT-PCR analysis. The qRT-PCR assays confirmed the microarray data (data not shown). Moreover, the *SOS1* expression level was verified separately in each hirsute woman (Fig.I A). Combining the *SOS1* qRT-PCR expression data we evidenced a statistically significant (*p* value < 0.05) *SOS1* over-expression in the hirsute patient group versus the control group (Fig.I B).

SOS1 protein is over-expressed in hirsute patients

Among the differentially expressed genes, *SOS1* showed the most significant change in mRNA expression level so we focused our attention on this gene. To evaluate if *SOS1* mRNA over-expression in the hirsute patients entailed SOS1 protein over-production, protein extracts from primary fibroblast cell cultures obtained from genital skin biopsies from the eight hirsute patients and the six controls were evaluated by western blot. We demonstrated that SOS1 protein was expressed at a higher level in the hirsute women than in the controls (Fig.II A). Densitometric analysis showed that SOS1 protein levels were higher in each patient when compared with the controls (Fig.II B). SOS1 protein over-expression was statistically significant (*p* value <0.05) in the hirsute patients group relative to the control group, as indicated by the densitometric analysis and standard deviations (Fig.II C). As shown in fig. II D, Pearson correlation analysis demonstrated a linear correlation between the percentage of SOS1 protein over-production obtained for each hirsute patient and the relative degree of hirsutism as indicated by the

Ferriman&Gallwey index score. This result indicated a strict correlation between SOS1 protein over-production and the Ferriman&Gallwey index score for each of the eight hirsute patients analyzed and the six healthy control women.
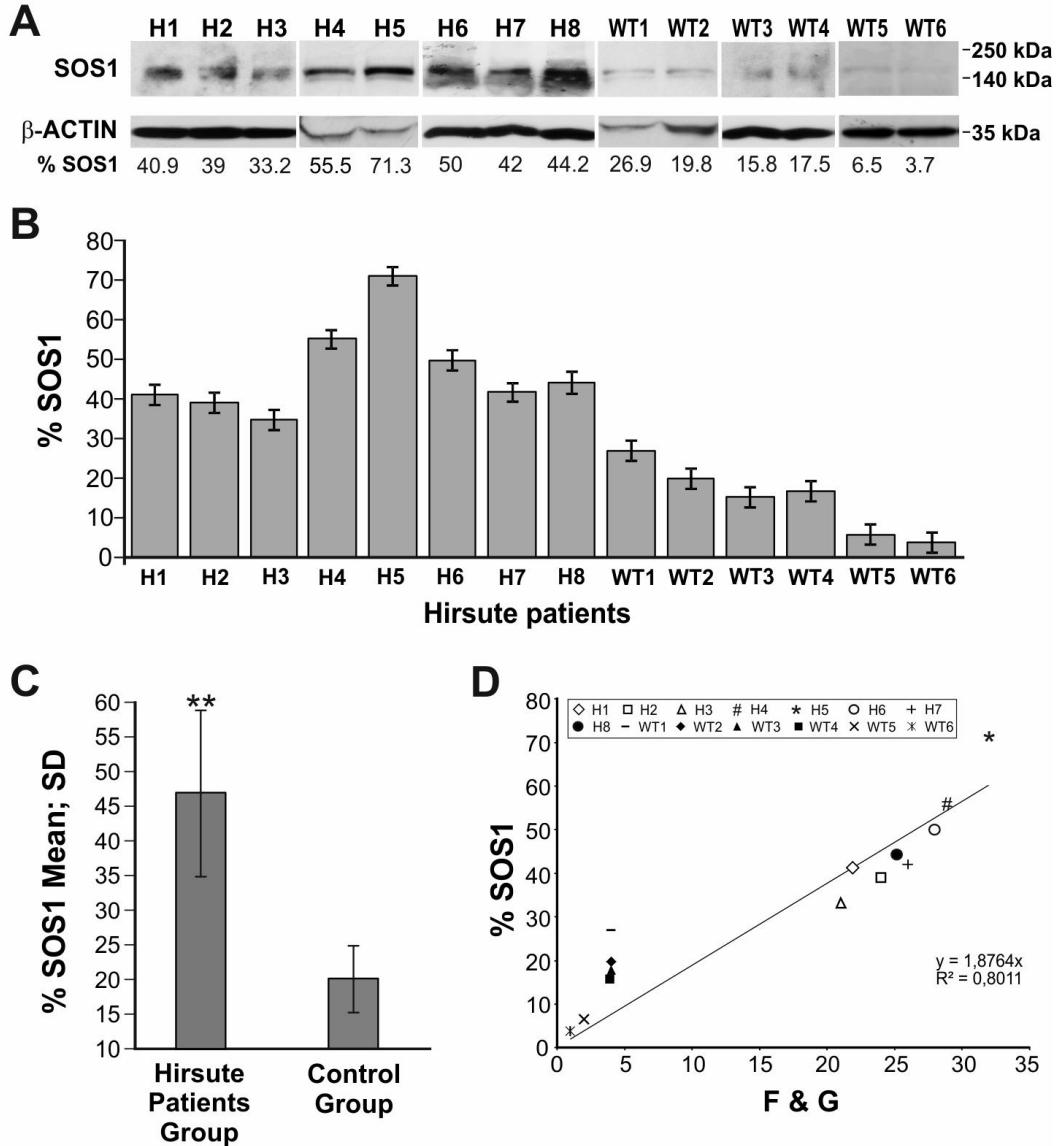

Fig.II SOS1 protein over-production in each hirsute patient assayed by western blot, relative densitometric analysis and correlation with the F&G values.

A) WB revealed that SOS1 protein was over-produced in each hirsute patient *vs* the relative controls. The SOS1 % derived from the densitometric analysis as described in the Materials and Methods section.

B) Densitometric analysis quantifying total SOS1 protein using β-actin as a control. Error bars represent the standard deviation of independent experiments.

C) The bar chart represents the SOS1 protein level in the hirsute patient group (n=8) *vs* the control group (n=6). The data are expressed as the mean and standard deviation of the percentage obtained from the densitometric analysis. The ** indicated a statistically significance (*p* value 0.004).

D) Pearson correlation analysis between the SOS1 protein level (expressed as a percentage) for each hirsute patient (obtained from densitometric analysis of the western blot assay) and the Ferriman&Gallwey index score of that patient.

Promoter and 3'UTR sequences analysis

To find functional SNPs that might modulate *SOS1* expression, we sequenced the promoter and 3'UTR in both the hirsute patients and the controls (data not shown). Ten SNPs were identified in the *SOS1* 3'UTR. Two of these SNPs have not been previously observed (g.139516 insCA; g.139544 delG).

Although some variants in the 3'UTR seem to have the potential to modulate the binding of transcription factors, *in silico* functional analysis predicted no functional abnormalities of the identified variants (data not shown).

SOS1 is over-expressed in DHT and T treated NHDF cells

Considering the SOS1 dys-regulation at the gene and protein level in the genital skin fibroblast of the selected hirsute patients, we hyphotized that SOS1 could represent a new paracrine/proliferative factor androgen induced, which could exert an important role in the hair follicle proliferation and cell cycle; for this purpose we reproduce an androgen-excess *in vitro* model using NHDF cell treated with different concentration of dhydrotestosterone (DHT) and testosterone (T) (miming an androgen induction) to assess the *SOS1* gene expression and protein levels and its effects on the Ras/Mapk pathway.

Firstly, we assessed *SOS1* gene expression levels in DHT- and T-treated NHDF cells at 3h, 6h and 24h by qRT-PCR using *β-actin* as internal control reference. We did not found a general changes in the *SOS1* expression levels, in DHT- and T-treated NHDF cells at 3h and 6h, with the exception for the 0.1nM DHT-treated NHDF cells for 3h in which we found a *SOS1* over-expression ($2^{-\Delta\Delta Ct}$=FC= +2.3) (Fig.III).
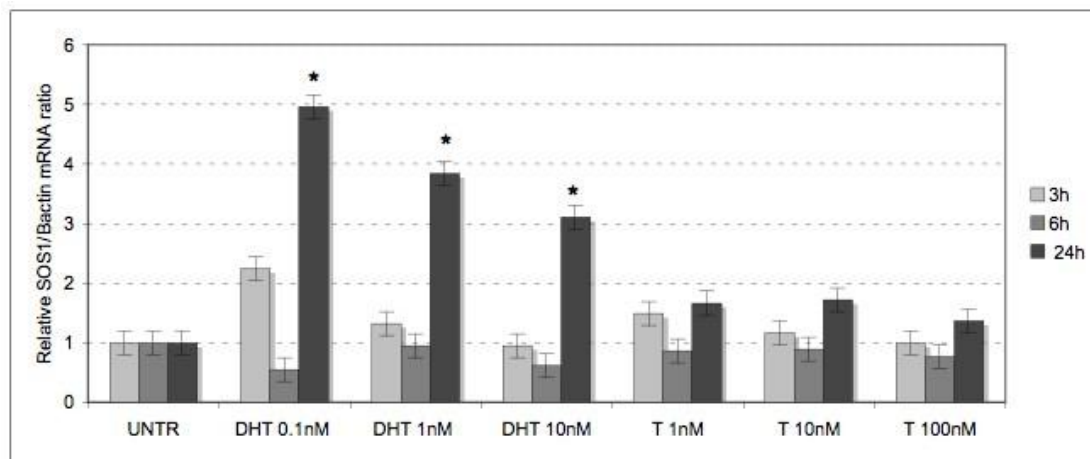

Fig.III The *SOS1* mRNA levels were analyzed by qRT-PCR separately in the DHT and T NHDF treated cell at 3h, 6 h and 24h. *SOS1* over-expression is indicated as relative *SOS1/* β-*actin* mRNA ratio. Error bars represent the relative standard deviation. The * indicated a statistically significance (p value < 0.05).

Interestingly, the difference in the *SOS1* mRNA was particularly evident in the DHT-treated NHDF cells at 24h than in the T-treated NHDF cells (Fig.III). In particular we found the highest *SOS1* over-expression ($2^{-\Delta\Delta Ct}$ =FC=+4.9) in

the 0.1nM DHT at 24h, and this over-expression level decrease gradually with higher concentration of DHT used for the NHDF treatments (1nM at 24h $2^{-\Delta\Delta Ct}$ =FC=+3.8; 10nM at 24h, $2^{-\Delta\Delta Ct}$ =FC=+3.1). These results were statistically significant with a *p* value < 0.05. On the contrary, a, we found did not found a significant *SOS1* changes in the gene expression levels in the T-treated NDHF cells.

In conclusion, we found an up-regulation in the *SOS1* mRNA after 24h/DHT treatment but not after T treatment. So, we may consider *SOS1* as a DHT inducible gene. In a second moment, we evaluated SOS1 protein over-production. Protein extracts from

T- and DHT-treated NHDF cell at 24h and NHDF untreated cells, used as control, were evaluated by western blot. The experiment revealed that SOS1 protein was expressed at higher levels in 24h/DHT-treated NHDF cells 0.1nM and 1nM, according to qRT-PCR data (Fig.IV A). Interestingly, in the 24h/DHT-treated cells, bicalutamide (BIC), a non-steroidal anti-androgen, reverted the DHT effects, lowering SOS1 protein amount to the basal levels (Fig.IV A). The increased SOS1 protein over-expression was statistically significant (*p* value <0.05) relative to the untreated cells, as indicated by the densitometric analysis and standard deviation (Fig.IV B).
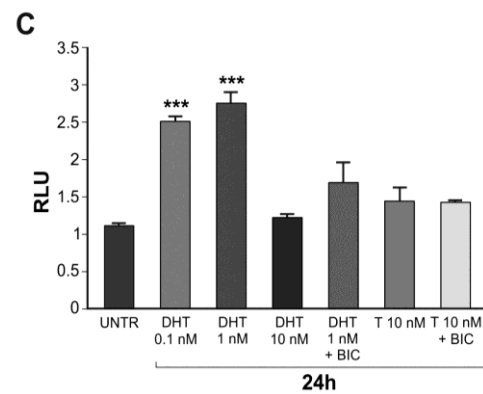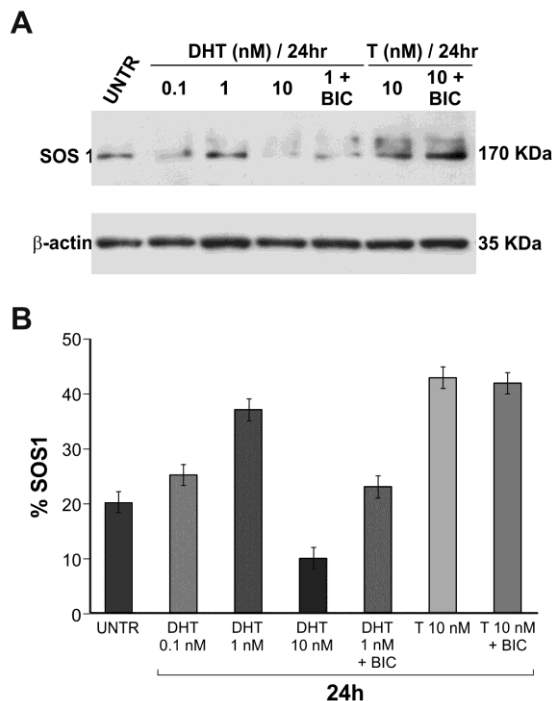

Fig.IV
Supplementary Table: Primer sequences for the *SOS1* promoter (A) and *SOS1* 3'UTR region (B).

SOS1 over-expression determined an increased Ras activity

Because SOS1 plays a critical role in the Ras/MAPK/ERK signaling pathway, the effect of the SOS1 modulation on Ras signaling was evaluated in the 24h treated and untreated NHDF cells.

Interestingly, Ras activity was low in serum starved control fibroblasts, but it increased 2,2 folds and 2,4 folds after 0,1nM and 1nM DHT treatment for 24h (*p* value<0,001) (Fig.IV C). These results were consistent with *SOS1* mRNA and protein over-expression

(Fig.III and Fig.IV A, B), indicating that 24h/DHT treatment activates a RAS-dependent signal transduction in dermal fibroblast. Moreover, bicalutamide (BIC) reverted the DHT effects, leading RAS activity to the basal levels. To date, this is the first demonstration that the DHT-induced SOS1/Ras pathway is androgen receptor dependent.

DISCUSSION

Although it is known that the sensitivity of the hair follicle to androgens may be crucial in the development of hirsutism (6-8), about the 22% of the women screened in our Unit of Endocrinology for this disorder have regular menstrual cycles with normal circulating adrenal and gonadal androgens and can therefore be classified as patient with "hirsutism of unknown cause". The new perspective to explain the pathogenesis of hirsutism may be related to a better understanding of the modifications in epithelial skin homeostasis which involves local factors that may amplify the androgen signal altering the hair follicle cycle (20). It is widely accepted that androgens are important regulators of human hair growth, but the molecular mechanism through which they act at the level of pilosebaceous unit are poorly understood. The current model for hormone action, developed for androgen action in human follicles, gives a central role to the mesenchyme-derived dermal papilla with androgens variably altering dermal papilla (DP) cell production of paracrine regulators like growth factors and extracellular matrix factors to coordinate the changes (21-22).

In the present study we have evaluated a selected group of severely hirsute women with the aim to call into question the molecular mechanisms underlying the variable response of the hair follicle to androgens and the hirsutism development. Our patients underwent genital skin biopsies to generate primary fibroblast cultures that were used to identify differentially expressed genes that might be involved in hirsutism pathogenesis. Among the differentially expressed genes *SOS1*, over-expressed, was further evaluated being involved in the insulin and EGF (epidermal growth factor) signal transduction pathways.

SOS1 is a guanine nucleotide-exchange factor (GEF) that couples receptor tyrosine kinases to Ras activation (23-28) and is thereby involved in the transduction of signals that control cell growth and differentiation (OMIM#182530).

Consider that *SOS1* was found over-expressed in the genital skin fibroblast of our hirsute patients and considering, as mentioned above, the important putative role of new actually unknown androgen-induced paracrine factorswhich could exert an important role in the hair follicle proliferation and cell cycle, we reproduced an androgen-excess *in vitro* model using NHDF cell treated with different concentration of T and DHT (miming an androgen induction) to assess the *SOS1* gene expression and protein levels and its effects on the Ras/Mapk pathway.

The key finding of our study is that the over-expression of *SOS1* mRNA is associated with over-production of SOS1 protein both in the genital skin fibroblast of hirsute patients and in the T or DHT treated NHDF cells. Our results suggest that the increase of SOS1 protein production could reflect

increased SOS1 signal through an activated RAS-ERK pathway. In fact, the activation of RAS is controlled by GEFs (like SOS1) that induce the release of GDP and allow them to function as molecular switches. The relative abundance of GTP in the cell compared to GDP results in the binding of GTP to the empty nucleotide-binding site and the activation of the GTPase. Mechanistically, it is possible that over-expression of *SOS1* induces increased GDP-GTP exchange and an increase in activated RAS, suggesting a potential role for the SOS1-RAS/EGFR pathway in the pathogenesis of hirsutism.

Studies evaluating the possible role of the *SOS1* gene in hirsutism are in their infancy. However, there is evidence to indicate that mutations in *SOS1* lead to a gain of function that correlates with constitutive activation of the RAS pathway, which supports the model proposed here. SOS1 has been identified as an etiologic agent for non-syndromic hereditary gingival fibromatosis (HGF) (29;OMIM#135300), a progressive hyperplasia of gingival tissues associated with hypertrichosis (30,31). Furthermore, mutations in the *SOS1* gene that cause a gain of function are implicated in a distinctive form of Noonan syndrome (OMIM#163950), in which the phenotype is characterized predominantly by ectodermal abnormalities including facial keratosis pilaris and curly hair (32). Similarly to the human phenotype, the K5-SOS-F transgenic mouse model of Noonan syndrome shows a dominant skin phenotype (33). These mice develop spontaneous skin tumors that resemble the lesions observed in transgenic mice expressing activated Ras in their hair follicles, thus suggesting that hyperkeratosis may result from activation of the Ras signaling pathway

by SOS. While there were no genomic variants or SNPs in the *SOS1* promoter region or in the 3'UTR that could be correlated with a functional effect by in silico prediction, some variants in the 3'UTR could modulate the binding of transcription factors (data not shown). However, further functional analysis and larger association studies are required to confirm this hypothesis.

In addition, because SOS1 is a signaling factor that regulates RAS activity, it is possible that over-production of the SOS1 protein activates the RAS-MAPK pathway and thereby stimulates skin stem cells to proliferate, altering hair growth as in hirsutism. A large number of studies have already demonstrated that the interfollicular epidermis and sebaceous glands contain unipotent progenitors that can preserve homeostasis of their respective tissue (34). These cells are organized in a particular structure and function as an epidermal proliferative unit. In skin, multipotent stem cells serve as a reservoir of cells for regeneration during the normal cyclic periods of hair growth, but they are also involved in the hyperproliferation of sebaceous glands (34) and in the repair of the interfollicular epidermis following wounding (35-37). Therefore, multiple signals have the potential to modulate multipotent skin stem cells and alter the hair growth cycle. In this context, SOS1 over-expression could serve as a proliferative signal that leads to increased RAS activity and hirsutism development.

In summary, our results on *SOS1* expression in genital fibroblasts may suggest that hirsutism may be the expression of a "RASopathy" wherein the RAS/MAPK pathway is an essential component in the regulation of the hair follicle cell cycle. This novel

preliminary observation has to be confirmed by further clinical and molecular studies exploring the variable development of hirsutism in response to androgen biological actions in order to develop new strategies in the anti-androgen treatment.

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