Intracrine androgenic apparatus in human bone marrow stromal cells

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Abstract

It was suggested that human mesenchymal stromal cells might contain an intracrine enzyme machinery potentially able to synthesize the cell's own supply of dihydrotestosterone (DHT) from dehydroepiandrosterone (DHEA) pro-hormone produced in the adrenal cortex in the reticular zone, which is unique to primates. Indeed, 3β-hydroxysteroid dehydrogenase (3β-HSD) and 5α-reductase enzyme proteins were expressed in resting mesenchymal stromal cells (MSCs) *in vitro*. However, the 'bridging' enzymes 17β-HSDs, catalysing interconversion between 17β-ketosteroids and 17β-hydroxysteroids, were not found in resting MSCs, but 17β-HSD enzyme protein was induced in a dose-dependent manner by DHEA. Quantitative real-time polymerase chain reactions disclosed that this was mainly due to induction of the isoform 5 catalysing this reaction in 'forward', androgen-bound direction ($P < 0.01$). This work demonstrates that the MSCs have an intracrine machinery to convert DHEA to DHT if and when challenged by DHEA. DHEA as substrate exerts a positive, feed-forward up-regulation on the 17β-hydroxy steroid dehydrogenase-5, which may imply that DHEA-DHT tailor-making in MSCs is subjected to chronobiological regulation.

Keywords: human bone marrow stromal cells ● dehydroepiandrosterone ● intracrinology ● hydroxysteroid dehydrogenase ● 5α-reductase ● dihydrotestosterone

Introduction

Only primates have in the cortex of adrenal glands a reticular zone, which produces dehydroepiandrosterone (DHEA) and its sulphate; lower animal species lack these precursor sex steroids. Peripheral tissues, like the male prostate, process DHEA further to active sex steroids. This research has led to clinical applications, 5α-reductase inhibitors, which are used in benign prostatic hyperplasia and prostatic cancer.

Bone cells contain steroid sulfatase and 17β-hydroxysteroid dehydrogenase (17β-HSD types 2 and 4). Aromatase has been described in human osteoblast-like cell line and 5α-reductase was reported in first passage human osteoblasts [1, 2]. Two groups have reported aromatase in human bone marrow mesenchymal stem cells (hMSCs) [3, 4] capable of differentiating towards osteogenic, adipogenic and chondrogenic lineages [5–7]. Aromatase is required for the conversion of testosterone to 17β-estradiol and of androstenedione to oestrone. Lack of type 2 3β-HSD and type 3 17β-HSD in hMSCs has also been reported, which casts some doubt if these cells contain a complete steroidogenic apparatus [8].

Based on the potential of osteoblasts to catalyse several intracrine reactions and of MSCs to catalyse at least aromatization, we suggested that bone marrow derived hMSCs might contain a more complete palette of enzymes for intracrine DHEA processing. We focused on the pathway necessary for the conversion of DHEA to dihydrotestosterone (DHT) (Fig. 1).

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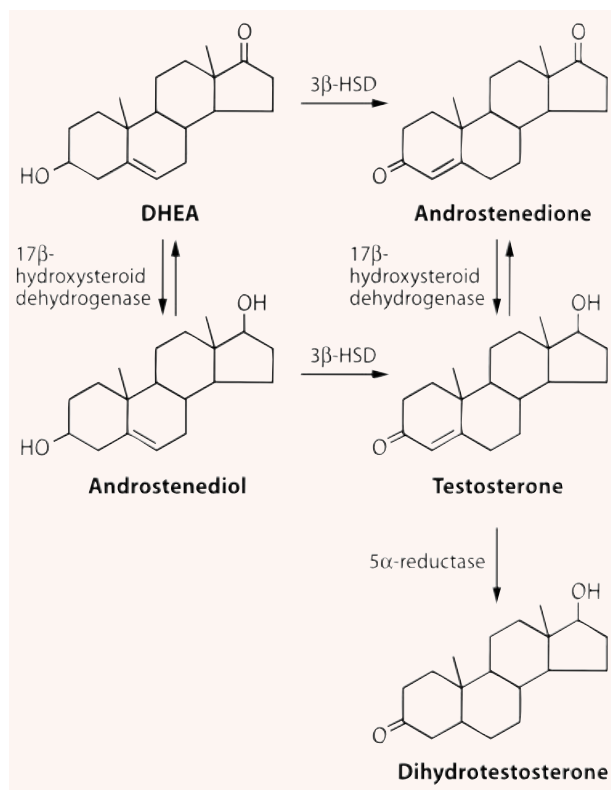Fax: +358-9-191+25218
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Fig. 1 Intracrine conversion of dehydroepiandrosterone (DHEA) to dihydrotestosterone (DHT). The enzymes involved are 3β-hydroxysteroid dehydrogenase (3β-HSD), 17β-hydroxysteroid dehydrogenase and 5α-reductase. These reactions are unidirectional except for the 17β-HSD catalysed reaction, which can run in forward and reverse directions in an enzyme isoform-dependent manner. Isoforms 1, 3 and 5 catalyse this reaction towards DHEA.

using commercial human 4–5 passage Poietics® MSCs (Lonza Walkersville, Inc., Walkersville, MD, USA), which were cultured in Poietics® Mesechymal Stem Cell Growth Medium (Lonza Walkersville, Inc.).

For experiments, $5–6 \times 10^3$ cells per cm^2 were plated to six-well plates containing cover slips. After 48 hrs, these cells were cultured ±10 or 100 μM DHEA (±1 nM dutasteride, types I and II 5-α-reductase inhibitor) or with 100 nM DHT (Fluka, St. Louis, MO, USA) for 6 hrs for mRNA expression and 24 hrs for immunocytochemistry.

Three fibroblast cell lines were established using explant culture method from synovial membrane samples. Briefly, tissue samples were minced to pieces and left overnight in RPMI-1640 medium (BioWhittaker, Liege, Belgium) containing 10% foetal bovine serum (BioWhittaker, Liege, Belgium) and 10% penicillin/streptomycin. Next day the media were changed and the concentration of antibiotics was decreased to 1%. The media were changed twice a week and after about 60% of the dish area was covered by monolayer of cells, the tissue pieces were removed and the cultures were allowed to grow to confluence. The cells were fibronectin and vimentin positive, whereas the proportion of cells positive for the CD163 macrophage marker was <1% (data not shown) and were used at passages 3–5 for immufluorescent staining.

Immunohistochemistry

Cells were fixed in 4% paraformaldehyde at +22°C for 20 min., washed in 10 mM phosphate buffered, 150 mM saline, pH 7.4 (PBS) with Triton X 2 × 10 min. followed by incubations in (*i*) 10% normal donkey serum in 0.1% bovine serum albumin in PBS for 60 min.; (*ii*) 4 μg/ml goat anti-human 3β-HSD IgG, 4 μg/ml goat anti-human 17β-HSD IgG, or 20 μg/ml goat anti-human 5α-reductase IgG (all from Santa Cruz Biotechnology, Heidelberg, Germany) for 60 min. and (*iii*) 10 μg/ml Alexa Fluor 568-labelled donkey anti-goat IgG (Molecular Probes, Eugene, OR, USA) for 60 min. Before mounting, nuclei were stained for 5 min. in 5 μg/ml 4′,6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA, USA). Non-immune goat IgG were used at the same concentration as and instead of the primary antibodies as negative staining controls. Cells were observed using Olympus motorized revolving AX 70 system microscope (Olympus Corp., Tokyo, Japan) coupled with 12-bits Sensicam digital image camera (PCO Imaging, Kelheim, Germany).

Quantitative real time-polymerase chain reaction (qRT-PCR)

Total RNA from cells was isolated using TRIzol reagent (Invitrogen, Paisley, UK) and mRNA using magnetic oligo(dT)$_{25}$ polystyrene beads (Dynal, Oslo, Norway). Messenger RNA concentrations were measured spectrophotometrically and complementary DNA (cDNA) was synthesized from 50 ng mRNA using oligo(dT)$_{12-18}$ primers and SuperScript enzyme, followed by RNase H treatment (SuperScript Preamplification System; Invitrogen). Quantitative RT-PCR was run in a LightCycler PCR machine using LightCycler FastStart DNA Master SYBR Green I kit (Roche, Mannheim, Germany) twice with each sample. Primers were designed with Primer3 (SourceForge, Mountain View, CA, USA), the sequences were searched with the NCBI Entrez search system, and sequence similarity search was done using the NCBI Blastn program. Primer sequences used were 5′-agctggacgtaagggactca-3′ and 5′-gtgggcgaggtattggtaga-3′ for 17β-HSD-1 (476 bp), 5′-tgcgtgagattctccagatg-3′ and

Materials and methods

Cell culture

Three primary MSC lines were established from healthy adult donors, who had given their informed consent and following a protocol approved by an institutional review board. From 20 ml bone marrow aspirate samples, the mononuclear cell fraction was isolated over a density gradient (Ficoll-Paque Plus, GE Healthcare, Uppsala, Sweden) and plated at 4×10^6/cm^2 in complete culture medium consisting of DMEM (low glucose, Sigma, St. Louis, MO, USA) and 10% foetal calf serum (Promocell, Heidelberg, Germany) with 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (all from Euroclone, Siziano, Italy). The non-adherent cell fraction was removed by washing after 72 hrs. Medium was changed twice weekly, and the passage 0 cells were harvested using TrypZean (Sigma) when subconfluent, usually 14 days after plating. The cells were replated at 1000/cm^2 in complete culture medium and passaged when subconfluent. Passages 3–5 were used in experiments. In addition, one set of experiments was done
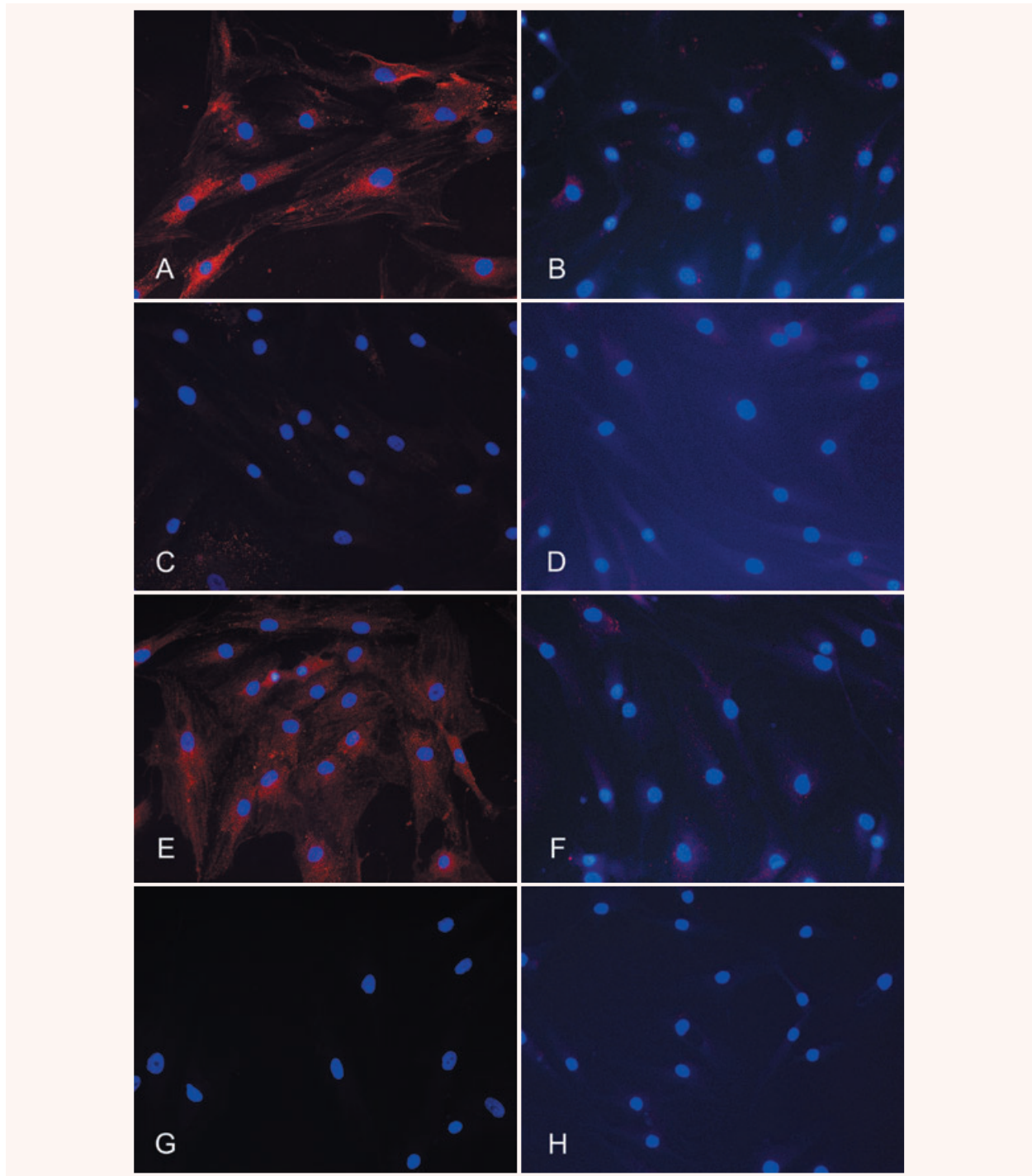
Fig. 2 Overlay figures of immunofluorescence staining of intracrine enzymes (in red colour) and 4′,6-diamidino-2-phenylindole nuclear counterstain (in blue colour). The first column shows unstimulated human bone marrow-derived mesenchymal stromal cells (MSCs) specifically labelled for 3β-hydroxysteroid dehydrogenase (**A**), 17β-hydroxysteroid dehydrogenase (**C**) and 5α-reductase (**E**). The second column shows the corresponding immunolabelling results of human synovial fibroblasts. Negative control staining with normal non-immune goat IgG performed to MSCs (**G**) and fibroblasts (**H**) confirmed the specificity of the staining results.

Table 1 Effect of dehydroepiandrosterone (DHEA) and dihydroxytestosterone (DHT) on 17β hydroxysteroid dehydrogenase (17β-HSD) mRNA expression. Data are normalized to average control group values.

	17β-HSD-1	17β-HSD-3	17β-HSD-5
Control	100.0	100.0	100.0
DHEA 1 μM	112.3 ± 16.2	143.9 ± 114.1	92.2 ± 6.0
DHEA 10 μM	93.8 ± 23.3	88.5 ± 13.7	103.8 ± 1.1
DHEA 100 μM	115.8 ± 10.3	156.9 ± 52.1	197.5 ± 14.3*
DHT 100 nM	91.3 ± 17.0	87.7 ± 11.0	114.4 ± 18.9

*Significantly different in comparison to control group, $P < 0.01$; n = 4.

5′-aatggcttgggagaaggttt-3′ for 17β-HSD-3 (358 bp), 5′-ccagttgactgca-gaggaca-3′, 5′-tcgctaaacaggacggattt-3′ for 17β-HSD-5 (233 bp) and 5′-tcacccacactgtgcccatctacga-3′ and 5′-cagcggaaccgctcattgccaatgg-3′ for β-actin (295 bp). For the qRT-PCR standard curve, the gene of interest was amplified in the PCR, extracted from an agarose gel, and cloned into the pCRII-TOPO vector (Invitrogen). After identification of the plasmid by restriction enzyme analysis and sequencing, the concentrations were determined spectrophotometrically, and serial dilutions were prepared for qRT-PCR analysis. The copy numbers of mRNA were determined from triplicates and normalized against $\times 10^{-6}$ β-actin genes. All values are provided as mean ± S.E.M. The data were processed by unpaired Student's t-test for statistical analysis.

Results

Studies of unstimulated hMSCs and fibroblasts

Immunofluorescence staining of unstimulated hMSCs showed the presence of 3β-HSD (Fig. 2A) and 5α-reductase (Fig. 2E), whereas no 17β-HSD was seen (Fig. 2C) (*n* = 4). Corresponding stainings of primary fibroblasts were negative (Fig. 2B, D and F). The staining controls confirmed the specificity of staining (Fig. 2G and H).

Although practically no 17β-HSD immunoreactive enzyme protein was found in immunofluorescence staining in resting hMSCs, there were some mRNA copies for the relevant 17β-HSD isoenzymes, 2.5 ± 1.2, 1.4 ± 0.4 and 298.3 ± 239.7 × 10^{-6} β-actin RNA copies for isotype-1, -3 and -5, respectively (*n* = 4).

Studies of stimulated hMSCs

As the findings on 17β-HSD were somewhat controversial further studies were done to assess its eventual sex steroid-mediated regulation. 17β-HSD immunoreactivity was in hMSCs induced in a

dose-dependent manner (Fig. 3A–D). Addition of 1 nM dutasteride in particular with the lower 10 μM DHEA increased this induction further (Fig. 3E) compared to 10 μM DHEA alone. DHT (Fig. 3G) and dutasteride by itself (Fig. 3H) did not induce 17β-HSD.

To supplement these immunohistochemical findings those 17β-HSD isoforms, namely 1, 3 and 5, which catalyse the intracrine reaction chain in a forward, DHT-bound direction were analysed. In accordance with the above mentioned immunohisto-chemical findings qRT-PCR disclosed a significant increase of the 17β-HSD-5 isoform (Table 1).

Discussion

Considering the 1-day circadian, 28-day menstrual, 9-month pregnancy and 0-to-80-year life cycle scale hormonal changes, peripheral tissues and cells are subjected to considerably different and changing concentrations of sex steroids *via* endocrine delivery. These differences in endocrine sex steroid delivery over time are further enhanced between different systemic concentrations in men and women. A local cellular and tissue specific intracrine system has evolved in primates to counteract ('buffer') the above mentioned sex steroid changes over time.

Human adrenal glands produce high amounts of DHEA and its sulphate pro-hormones, which are used at least in some peripheral tissues to tailor-make active sex steroids [9, 10]. Aromatase has been studied in hMSCs [3, 4, 11]. It is now reported that hMSCs are self-sufficient as to the enzymatic apparatus responsible for conversion of DHEA to oestrogen precursors (androstenedione and testosterone). Alternatively, they can use testosterone to produce DHT in a 5α-reductase-catalysed reaction.

Resting hMSCs displayed 3β-HSD and 5α-reductase, but not 17β-HSD, which leaves a gap between the initial early and late terminal conversion reactions. As this did not seem to make sense, DHEA and DHT stimulation experiments were performed to assess eventual substrate and end product regulation. Dose–response studies on the effect of DHEA on 17β-HSD were done using 0, 1, 10 and 100 μmol concentrations of DHEA, the two last mentioned DHEA concentrations were also tested in the presence of dutasteride, which is inhibitor of the 5α-reductase. Dutasteride inhibits conversion of testosterone (produced from DHEA by 3β-HSD and 17β-HSD) to DHT. These results show that the 100 μmol DHEA concentration, shown also using some other cells to be effective *in vitro* [12], increases the isotype 5 of 17 β-HSD significantly. Because the absolute copy numbers of this isotype were also in absolute terms highest (and most variable of the three), this is likely also to be the biologically most significant isotype in the DHEA-induced conversion of DHEA-substrate in the direction to DHT end product. Based on staining it was evident that 17β-HSD-inducing effect of 10 and 100 μmol DHEA was further enhanced by dutasteride, which inhibits the final conversion of testosterone to DHT. This suggests that the intermediates between DHEA and testosterone, which increase in the presence of dutasteride, help to induce 17β-HSD.
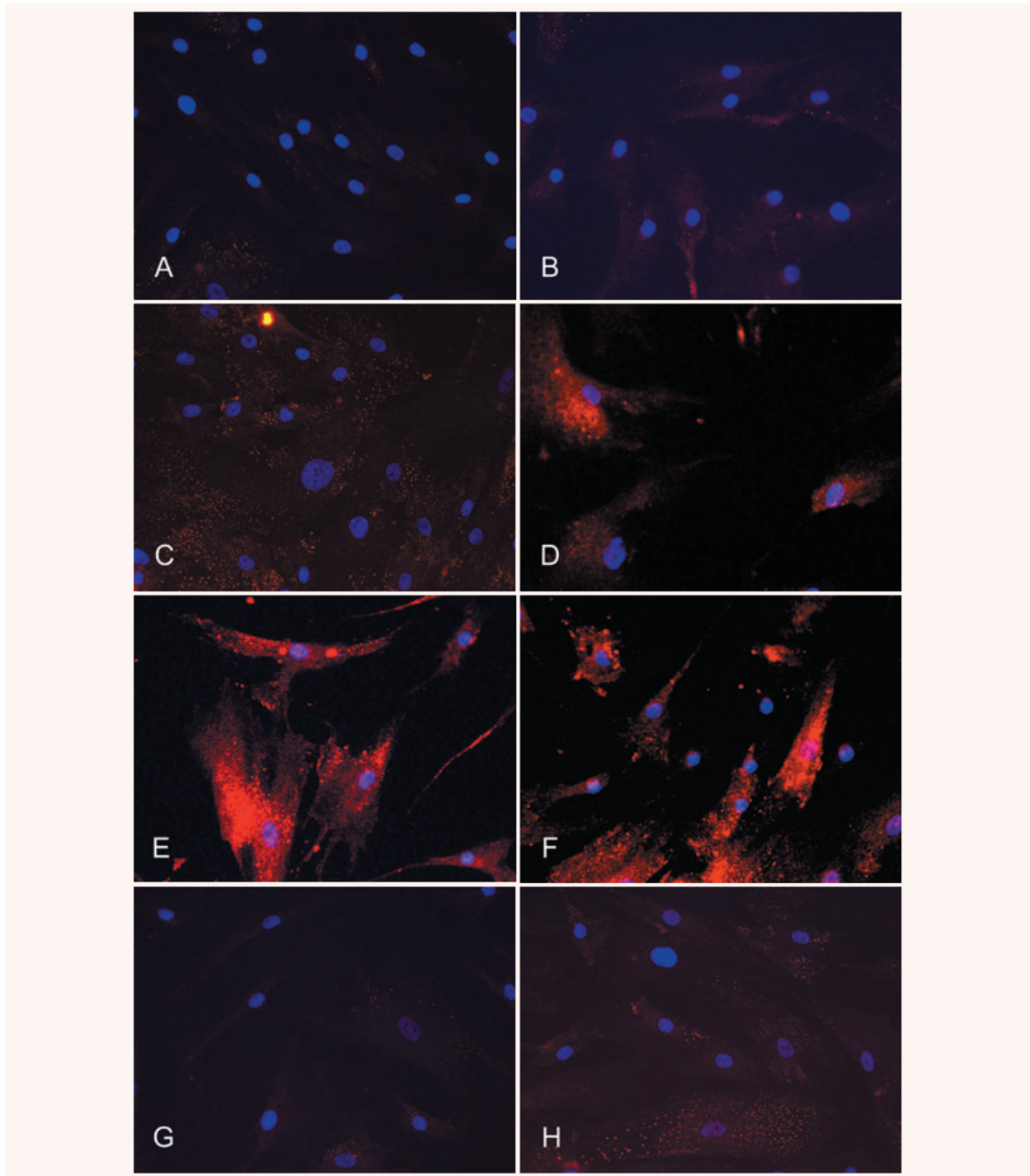
Fig. 3 Merged immunofluorescence of 17β-hydroxysteroid dehydrogenease (red) and nuclear DAPI (blue) of mesenchymal stromal cells cultured without dehydroepiandrosterone (**A**), with 1 μM (**B**), 10 μM (**C**) and 100 μM (**D**) dehydroepiandrosterone. (**E**) and (**F**) show the effects of 10 and 100 μM dehydroepiandrosterone, respectively, in the presence of 1 nM dutasteride (which inhibits the conversion of testosterone to dihydrotestosterone [DHT]). Culture with 100 nM DHT (**G**) or 1 nM dutasteride alone (**H**) did not induce 17β-hydroxysteroid dehydrogenease.

17β-HSD reaction is bidirectional (Fig. 1). Antibodies used for immunolabelling of 17β-HSD are polyclonal and not isotype specific. Therefore, the mRNAs of those isoforms catalysing the conversion in a forward, DHT-bound direction were analysed and 17β-HSD-5 was found to be the isoform responsible for the appearance of the immunoreactive enzyme in the presence of DHEA substrate.

DHEA and DHEA-sulphate concentrations show two peaks, one during foetal and neonatal period and another at the age of 20–25 years, which is followed by declining DHEA concentrations during adrenopause; only very low levels remain at the age of 80 years. Presence of an intracrine androgenic apparatus able to convert DHEA to substrates for oestrogen synthesis and/or DHT suggests that hMSCs are subjected to an important age-dependent or chronobiological regulation. This ability may have important intra-, auto- and paracrine consequences for the MSCs and for the cells located in their vicinity.

Intracrine metabolism of DHEA to DHT has been mostly studied in prostate cancer. These studies have disclosed that DHT can be produced for both paracrine and intracrine purposes. In healthy prostate gland, non-epithelial stromal cells utilize type 2 isotype of 5α-reductase supposed to produce DHT for export to tubuloepithelial prostate cells. Upon transformation to cancer cells, these tubuloepithelial cells become autonomous in this respect by starting to express type 1 isotype of 5α-reductase so that they are able to produce their own DHT. This represents a change from stromal based, paracrine androgen receptor axis (where the cells are regulated by androgens produced by other close-by cells) to an autocrine pathway (where the cells produce androgens for autostimulation) [13]. In this respect, MSCs, when provided with the DHEA pro-hormone substrate, also seem be potentially self-sufficient because they contain all the three intracrine enzymes needed for conversion of DHEA to DHT, which, however, needs to be confirmed in future hormonal conversion and tracing studies.

MSC *in vitro* are expanded fibroblastic colonies, whereas *in vivo* these cells are rare and may not make a significant contribution to the sex steroid supply. One may therefore ask, what is the clinical relevance of the current findings? Instead, it was suggested that such anabolic repair cells would require DHT so that there is a cell- and/or site-specific demand and that they therefore could contain an enzymatic apparatus able to respond to such a demand. Utilizing intracrine enzymes they could produce DHT inside the cell cytoplasm and nucleus for their own needs (intracrine, and possibly to some extent also auto- and paracrine mechanism of action, in contrast to endocrine mechanism of action). Interestingly, comparison of MSCs with fibroblasts disclosed that fibroblasts seem to lack such an intracrine enzymatic apparatus.

The evidence provided for the intracrine DHEA metabolism is still preliminary in that sense that only the enzyme mRNAs and enzyme proteins driving the intracrine pathway from DHEA to DHT are shown, not the intracrine metabolism *per se*. However, because the role of these specific enzymes is widely acknowledged in all differentiated cells containing them, they are hardly vestigial remnants of evolution and probably fulfil such an intracrine role also in the progenitor MSCs. Also the eventual significance of DHEA converting intracellular machinery in hMSCs requires further studies. Because sex hormones influence stem cells and their differentiation capability [14], it is possible that expression of such an intracrine enzymatic machinery provides MSCs a certain autonomic advantage (or buffering capacity) enabling them to maintain their undifferentiated state (stemness) or differentiation capability towards different cell lines (pluripotency) even in a constantly changing systemic or local hormonal environment affected by circadian, menstrual, pregnancy-related and other chronobiological changes.

Acknowledgements

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