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Research Article

Stemness markers characterize IGR-CaP1, a new cell line derived from primary epithelial prostate cancer

Anne Chauchereau[a,h,*], *Nader Al Nakouzi*[a,h], *Catherine Gaudin*[a,h], *Sylvestre Le Moulec*[a,h], *Daniel Compagno*[b], *Nathalie Auger*[c,h], *Jean Bénard*[c,h], *Paule Opolon*[d,h], *François Rozet*[e], *Pierre Validire*[f], *Gaëlle Fromont*[g], *Karim Fizazi*[a,h]

[a]*Prostate Cancer Group, INSERM U981, Institut Gustave Roussy, Villejuif, F-94805, France*
[b]*Laboratory of Prostate Cancer, Dep. Química Biológica-University of Buenos-Aires-FCEyN, Buenos-Aires, Argentina*
[c]*Department of Medical Biology and Pathology, Institut Gustave Roussy, Villejuif, F-94805, France*
[d]*UMR8121, Institut Gustave Roussy, Villejuif, F-94805, France*
[e]*Department of Urology, Institut Mutualiste Montsouris, Paris, F-75014, France*
[f]*Department of Pathology, Institut Mutualiste Montsouris, Paris, F-75014, France*
[g]*Department of Pathology, CHU-University of Poitiers, Poitiers, F-86000, France*
[h]*University Paris-Sud 11, France*

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ABSTRACT

Deciphering molecular pathways involved in the early steps of prostate oncogenesis requires both *in vitro* and *in vivo* models derived from human primary tumors. However the few recognized models of human prostate epithelial cancer originate from metastases. To date, very few models are proposed from primary tumors and immortalizing normal human prostate cells does not recapitulate the natural history of the disease. By culturing human prostate primary tumor cells onto human epithelial extra-cellular matrix, we successfully selected a new prostate cancer cell line, IGR-CaP1, and clonally-derived subclones. IGR-CaP1 cells, that harbor a tetraploid karyotype, high telomerase activity and mutated TP53, rapidly induced subcutaneous xenografts in nude mice. Furthermore, IGR-CaP1 cell lines, all exhibiting negativity for the androgen receptor and PSA, express the specific prostate markers alpha-methylacyl-CoA racemase and a low level of the prostate-specific membrane antigen PSMA, along with the prostate basal epithelial markers CK5 and CK14. More importantly, these clones express high CD44, CD133, and CXCR4 levels associated with high expression of α2β1-integrin and Oct4 which are reported to be prostate cancer stemness markers. RT-PCR data also revealed high activation of the Sonic Hedgehog signalling pathway in these cells. Additionally, the IGR-CaP1 cells possess a 3D sphere-forming ability and a renewal capacity by maintaining their CSC potential after xenografting in mice. As a result, the hormone-independent IGR-CaP1 cellular clones exhibit the original features of both basal prostate

* *Corresponding author*. Prostate Cancer Group, INSERM U981, Institut Gustave Roussy, 114 rue Edouard Vaillant, Villejuif, F-94805, France. Fax: +33 1 42 11 60 94.

E-mail addresses: anne.chauchereau@igr.fr (A. Chauchereau), nader.nakouzi@igr.fr (N. Al Nakouzi), catherine.gaudin@igr.fr (C. Gaudin), sylvestre.lemoulec@gmail.com (S. Le Moulec), danielcompagno@qb.fcen.uba.ar (D. Compagno), nathalie.auger@igr.fr (N. Auger), jean.benard@igr.fr (J. Bénard), paule.opolon@igr.fr (P. Opolon), francois.rozet@imm.fr (F. Rozet), pierre.validire@imm.fr (P. Validire), g.fromont@chu-poitiers.fr (G. Fromont), karim.fizazi@igr.fr (K. Fizazi).

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tissue and cancer stemness. Tumorigenic IGR-CaP1 clones constitute invaluable human models for studying prostate cancer progression and drug assessment *in vitro* as well as in animals specifically for developing new therapeutic approaches targeting prostate cancer stem cells.

Introduction

Prostate cancer is the second leading cause of cancer-related deaths in men in North America and Europe. Nowadays, there is still no cure available for patients with advanced disease especially when hormone independence emerges [1]. Cell cultures established directly from primary tumors from patients are powerful research resources for studying cancer cell biology and for developing new strategies against cancer. However, human prostate cells are known to be one of the most difficult tissues to develop in a continuously growing culture especially while maintaining hormone dependency. Most *in vitro* or xenografted models of prostate cancer have been established from metastases [2,3]. The immortalization of normal human prostate cells, either epithelial or stromal, has been proposed to extensively investigate the early genetic events that give rise to epithelial prostate cancer progression. However, to date, these artificial models have been unable to identify the cells that initiate prostate cancer. Clearly, due to difficulties inherent to *in vitro* culture, models directly derived from primary tumors are still lacking. In spite of huge efforts, only two continuously cultured human primary epithelial prostate cancer cell lines have been established, the E006AA [4] and the HH870 [5] cell lines but they have not been documented so far, except for a recent study of AR signalling in the E006AA cell line [6]. A third human prostate cancer model was recently established *in vitro* from a trans-rectal prostate needle biopsy specimen but it was unable to grow in nude mice [7]. Currently, prostate cancer models, directly derived from primary tumors, enabling both *in vivo* and *in vitro* approaches and representing the early stages of this cancer are still lacking.

Regarding homeostasis of prostate tumor cells, there is mounting evidence that prostate cancer results from a hierarchical model originating from rare cancer stem cells (CSC) that enables tumor maintenance [8]. Based on this model, it has been suggested that androgen-independent stem cells give rise to two types of cells, stem cells and androgen-independent transit-amplifying cells, capable of differentiating into luminal cells. Prostate cancer stem/progenitor cells may exhibit similar characteristics to those of normal stem cells likely localized in the basal cell compartment [9]. Collins et al. [10] first identified and isolated prostate stem cells from primary tumors. It was subsequently shown that these cells express CD44, α2β1-integrin and CD133, normal prostate epithelial stem cell markers [11,12]. Recently, telomerase-immortalized basal prostate epithelial cell (HPET) lines were obtained which provide a constant supply of cells while simultaneously maintaining the differentiation characteristics of the original tissue [13]. Clonally-derived HPET cells reconstitute the original human tumor *in vivo* and differentiate into the three prostate epithelial cell lineages, indicating a common stem/progenitor cell [14]. More recently, prostatic epithelial RC-165N/hTERT cells were obtained which exhibit a stable stem cell phenotype $CD133^+/CD44^+/\alpha2\beta1^+/34\beta E12^+/CK18^+/p63^-/AR^-/PSA^-$ and the ability to differentiate into AR-positive cell types *in vitro* [15,16]. Most of these cancer stem cells were identified using surface markers that recognize their corresponding normal tissue stem and progenitor cells, thus strengthening the resemblance between tumor development and normal prostate organogenesis. Until now, these markers essentially pinpoint cell populations with CSC characteristics.

Here, we describe the establishment and characterization of a new prostate cancer cell line and cell-derived clones from a primary tumor. These cells show phenotypic features of prostate epithelial basal cells, exhibit the original features of prostate tissue and retain cancer stem cell properties. Thus, our new IGR-CaP1 models may help develop new therapeutic approaches that target prostate cancer stem cells.

Materials and methods

Primocultures and cell cultures

After mechanical dissociation of human prostate tumor tissue and counting, cells were seeded on an extracellular matrix (ECM) of human epithelial origin, IGR-XC, and further cultured in RPMI medium supplemented with 10% FBS (foetal bovine serum, Gibco), penicillin-streptomycin antibiotics and fungizone. This human ECM, developed at the *Institut Gustave Roussy*, Villejuif, France (IGR-XC, US patent 7,476,496), prevents anoikis and enables epithelial cells to anchor onto the substrate [17]. Primoculture conditions combined IGR-XC ECM and proline-depleted RPMI medium to prevent fibroblast contamination [18]. The cells were incubated at 37 °C with 5% CO_2 until they reached semi-confluence and were then trypsinized. After maintaining these culture conditions for 20 passages, cells were allowed to grow onto plastic flasks in regular RPMI medium containing 10% FBS and antibiotics. The renowned prostate cancer cell lines LNCaP and PC3 were cultured in RPMI-1640 containing 10% FBS and MDA-PCa-2b cells, as previously described [19]. To re-establish *in vitro* cultures after xenografting into nude mice, xenografted tumors ($n=5$) were collected when they attained 300 mm^3. Tumor cells were dissociated by a 30 min digestion at 37 °C with Collagenase H/DNAse I (Roche) in culture medium, as previously described [20] and plated in 6-well cell culture dishes in classic culture medium. Forty-eight hours after, the medium was replaced by proline-depleted RPMI medium and cells were cultured for 5 passages.

Clinical summary

The prostate primary tumour samples were collected from the Institut Mutualiste Montsouris (Paris, France) from 3 patients in accordance with protocols approved by the local ethics committees. The prostate cancer tissue that yielded the IGR-CaP1 cell line was obtained from a 58-year-old Caucasian French patient who had undergone a radical prostatectomy for clinically localized prostate cancer. At diagnosis, the serum PSA level was 5.6 ng/ml.

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Pathological analysis of the prostatectomy specimen revealed a massive bilateral posterior tumor confined to the prostatic capsule which was a moderately-differentiated adenocarcinoma with a Gleason score of 7 [4(70%) + 3(30%)]. The clinical stage assigned was pT2c Nx.

Cell growth kinetics

Cell growth kinetics was determined by counting the number of viable cells at regular intervals. After seeding in triplicate at 4000 cells/well in 12-well plates in normal culture medium or in medium containing 10% charcoal-stripped FBS, cells were trypsinized daily, stained with trypan blue and counted. The doubling time was calculated from the regression equation of the curve. For the hormone-dependent growth assay, cells were seeded at 10 000 cells/well in 96-well plates. After 24 h, the culture medium was replaced with phenol red-free medium containing 10% charcoal-stripped FBS and dihydrotestosterone (DHT) was added or not, at a final concentration of 10^{-9} M. The medium was replaced each day. After 72 h, cell survival was measured with the WST1 test (Roche).

Telomerase assay

Telomerase activity was measured by using the Biomax Telomerase detection kit (Biomax Inc., MD, US) based on a quantitative real-time telomeric repeat amplification protocol, according to the manufacturer's recommendations. Telomerase activity was determined through its ability to synthesize telomeric repeats onto an oligonucleotide substrate in cellular extracts and the resulting extended products were amplified by PCR (35–40 cycles) using the DNA SYBR Green fluorochrome and measured on a 7900HT Fast Real-Time PCR System (Applied Biosystems). Telomerase activity was quantified according to the manufacturer's recommendations against a standard curve that had an R^2 of 0.98.

DNA sequencing for analysis of TP53 mutation

Sequencing was performed from cDNA as previously described [21]. Two different DNA preparations obtained from different cell aliquots showed the same Tp53 mutation.

Karyotype

Metaphases were harvested after a 2.5-h colchicine block. Chromosome spreads were obtained according to previously described techniques [22]. Karyotypes were established on more than 10 metaphases after R-banding according to the standardized human karyotype.

Short tandem repeat DNA typing (STR typing)

Genomic DNA extracted from the cells was prepared using the QIAamp DNA Micro kit (Qiagen) and was quantified using real-time PCR technology. The short tandem repeat (STR) analysis was conducted using the multiplex-PCR-based Identifiler amplification kit (Applied Biosystems) on 1 ng of genomic DNA in which 16 STR loci were simultaneously co-amplified. Automated DNA fragment analysis was performed on an ABI3130xl Genetic Analyser. Fluorescent data were collected and analyzed using GeneMapper 3.2 ID-specific genotyping software (Applied Biosystems). The resulting profile showed the assigned allele values corresponding to the number of repeat units identified for each locus.

In vivo tumorigenicity assay

Six-week-old male athymic nude mice (NC-nu/nu) (Janvier, France) were used in conformity with the Guidelines of the French Government regarding operative procedures and animal care. IGR-CaP1 cells (10^7) were subcutaneously injected into the dorsal side without matrigel. Orthotopic injections were performed, as previously described [23]. Briefly, the prostate of each anesthetized mouse was exposed via a midline laparotomy incision and 10^6 cells in 5 µl PBS were directly injected into the prostate.

Western blot analysis

Western Blot assays were performed on 50 µg of whole cellular lysates. Blots were probed with anti-AR (N-20) or anti-PSA antibody (C-19) from SantaCruz, or anti-Oct4 antibody (Chemicon). Immunoblot analyses were developed using the enhanced chemoluminescence-based detection kit (Pierce).

Flow cytometry

FACS analysis was used to determine differentiation marker expression and to sort cells. For cytoplasmic or nuclear proteins, permeabilization in 0.25% triton X-100 was performed before labelling. The following antibodies were used: anti-human EpCAM-PE (clone EBA-1, Becton Dickinson), PSMA-FITC (clone 107-1A4, MBL medical), CK5/8-FITC (clone 5F173, US-Biological), CK8-FITC (clone B22.1, GeneTex), CK14-FITC (clone 2Q1030, US-Biological), CK18-FITC (abcam), CD24-FITC (clone ML-5, BD pharmingen), CD44-FITC (clone G44-26, BD Biosciences), CD133-APC (AC133, Miltenyi-Biotec), CXCR4-PE (also named CD184-PE, clone 12G5, BD Biosciences), P504S (AMACR) (2A10F3, Santa Cruz). The corresponding isotype control antibodies were included in each staining condition. For indirect labelling, purified mouse IgG2b and rabbit IgG (R&D System) were used as isotype controls and Molecular Probes' Alexa Fluor 488 (1:200) were used as secondary antibodies. Samples were analyzed with the FACS Calibur cytometer (Becton Dickinson).

TaqMan real-time quantitative reverse transcription-PCR analysis

Total RNA was extracted from cell lines using the RNeasy Midi kit (Qiagen) and 1 µg of RNA was reversed transcribed using random hexamers (Applied Biosystems). Quantitative real-time PCR was performed with the ABI Prism 7900 Sequence Detection System (Applied Biosystems) using 5 µl of 1:20 diluted cDNA in a final volume of 25 µl according to the manufacturer's recommendations. The reference and sequence of PCR primers and probes were designed by Applied Biosystems (see Supplementary Material) and used according to the manufacturer's recommendations. The amount of sample RNA was normalized by amplification of an endogenous control (18S). In each experiment, the relative quantification of the transcripts was derived using the standard curve method. Results obtained in the IGR-CaP1 cells were compared to that found in LNCaP cells.

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Immunohistochemistry (IHC)

The different tissue specimens (primary tumors and potentially metastatic organs) from mice were fixed in Finefix (Milestone Medical), and then paraffin sections (4 μ thick) were processed and stained with hematoxylin–eosin–saffranin (HES). Tumor and organ sections were incubated with anti-pan-CK (AE1/AE3/PCK26 (APK), Ventana Medical Systems), anti-p53 (Ventana Medical Systems), anti-PSMA (3E6) or anti-PAP (PASE/4LJ) from Diagnostic BioSystems, anti-Ki67 (Zymed), anti-CK14 (LL001, R&D Systems) antibodies. All sections were analyzed using a Zeiss Axiophot microscope and a SensiCam PCO digital camera. Representative views were taken at 100× magnification.

Immunofluorescence microscopy

Cells were fixed in 4% formaldehyde, washed and then incubated with goat serum solution (1:100) and anti-human α2β1-integrin antibody (BHA2.1, Chemicon) followed by incubation with Alexa-Fluor 488 antibody (Molecular Probes). Nuclei were stained with Dapi vectashield mounting reagent (Vector Laboratories). Images were acquired on a Zeiss Axioplan 2 microscope.

Spheroid soft agar assay

Prostate spheroid cultures were made from IGR-CaP1 cells (P30). Cells were seeded at 10 000 cells/well in 12-well plates in 0.35% agarose in growth medium overlaid on a base of 0.8% agarose. Cultures were fed every 4–5 days for 2 weeks until colonies were formed. The 3D cultures were performed in classic RPMI medium supplemented with FBS. Cells grown as nonadherent spherical clusters were fixed in Finefix (Milestone Medical), collected using the cytoblock kit (Thermo laboratories) and embedded in paraffin. Then paraffin sections (4 μ thick) were processed and stained with HES.

Results

The IGR-CaP1 cell line is a prostate cancer epithelial cell line

In a combination of ECM of human epithelial origin, IGR-XC and medium–which avoids fibroblast overgrowth–primary tumor fragments from three patients with localized disease were seeded and only one tumor fragment, from a 58-year-old prostate cancer patient, generated stabilized tumor cells after 20 subcultures *in vitro*. These cells were spontaneously immortalized since they were able to grow on plastic dishes in classic culture medium while retaining their proliferative capacity in a continuous long-term culture (up to 50 passages). They grew as adherent cells with epithelial cell morphology (Fig. 1A) and expressed the epithelial cell adhesion molecule EpCAM (Fig. 1B) as compared with the isotypic control or EpCAM-negative human fibroblasts IMR90 (not shown). They also stained positively for cytokeratin, as observed in the original tumor (Fig. 1C–D), confirming the epithelial origin of this cell line. The absence of chromogranin A expression in IGR-CaP1 cells led us to rule out the hypothesis of neuroendocrine cells while the lack of the mesenchymal markers STRO-1, CD73 and CD105 (not shown) and proline-depleted medium used to establish the cell line rather suggested that these cells did not correspond to mesenchymal cells either.

IGR-CaP1 cell identification by genomic profiling analyses

To better characterize the cell line, genotyping of the complete Short Tandem Repeat (STR) profile was performed on total DNA from the IGR-CaP1 cell line (Table 1). This technique allowed definitive cell line authentication. The profiles confirmed the complexity of the karyotype (Fig. 2A) and showed the absence of the Y chromosome on the amelogenin locus. We observed the same STR profile between DNA prepared from IGR-CaP1 cells at passages 10 and 50, showing that chromosome alterations were conserved throughout cultures. Since establishing a primary cell culture may generate cellular heterogeneity, we undertook clonal selection from the parental IGR-CaP1 cells by limit dilutions and obtained nine clonally-derived clones. To definitively identify two of them, named 3A11 and 3C11, the STR profile was determined and showed some minor differences compared with that of the parental IGR-CaP1 cell line (Table 1). Allelic changes were observed at two loci vWA and D18S51, for the 3A11 derived clone and at 5 loci D2S1338, D19S433, D18S51, D5S818 and FGA for the 3C11 derived clone.

Karyotypic analysis was performed on the IGR-CaP1 cell line at passage 29 using a conventional R-banding technique. The analysis showed a complex tetraploid karyotype with 86–91 chromosomes, including numerical and structural rearrangements (Fig. 2A). The karyotype was determined to be 86~89 <4n>,XX,+der(X),+der(X),-Y,-Y, der(1)*t*(1;?)(p32;?), add(2)(q3?2), −3,der(3)*t*(3;?)(p10;?), −4, −4, −5, −5, −6, −6,ins(7;?)(p15;?),+8,+8,+11, +11, −13, −13, add(13)(q31), −14, −15, −15,del(18)(q22),del(18(q22),+20,del(20)(q12),+del(20)(q12),+mar inc[cp15].

IGR-CaP1 cells spontaneously express high telomerase activity and show malignant features in vitro

Telomerase, the enzyme responsible for replicating telomeres, is expressed at a low level in most normal tissues and becomes activated during tumorigenesis. As telomerase expression can itself induce immortalization [24], we measured the telomerase activity in IGR-CaP1 cell extracts using a quantitative PCR system targeting telomere extension products. The spontaneously immortalized IGR-CaP1 cells exhibited high telomerase activity compared with normal lymphocytes (PBL) (Fig. 2B). However, this activity was lower than that measured in the PC3 cells derived from a metastatic site.

Since mutations in the tumor suppressor gene *Tp53* are frequently associated with chromosome instability, Tp53 expression was examined in IGR-CaP1 cells. Immunohistochemical analysis showed high Tp53 protein expression in the parental tumor and in the derived cells (Fig. 2C–D). Tp53 expression in the IGR-CaP1 cells (P16) was confirmed by Western Blot analysis with the anti-Tp53 DO-7 antibody (not shown). High Tp53 expression was assumed to correspond to the stabilized mutated *Tp53* gene product. Sequencing did indeed reveal a missense mutation at the nucleotide A377G corresponding to a change at codon Y126C.

Cell growth and kinetics

The IGR-CaP1 cells grew rapidly in classic culture medium conditions with a doubling time of 43 h. Similar cell kinetics were observed in medium containing charcoal-stripped FBS (Fig. 3A). Treatment with DHT had no effect on IGR-CaP1 cell nor

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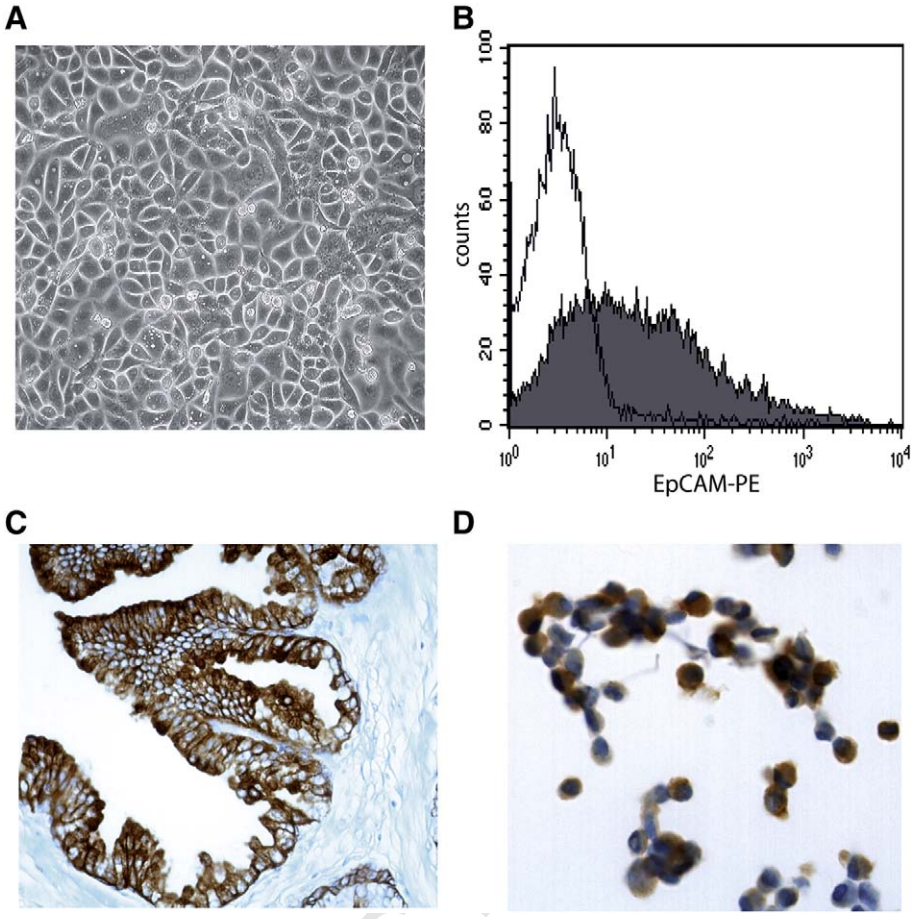

Fig. 1 – Morphology and epithelial features of the IGR-CaP1 cell line. (A) Photomicrograph of the human primary PCa cell line IGR-CaP1 (passage 29) shows typical epithelial morphology. (B) Expression of EpCAM was evaluated by FACS. (C-D) Cytokeratin expression was analyzed by IHC with anti-pan cytokeratin antibody (APK) in the initial tumor (C) and in IGR-CaP1 cells at passage 16 (D).

Table 1 – STR analysis for identification of IGR-CaP1 cells and the two clonally-derived clones 3A11 and 3C11.

Locus ID	IGR-CaP1	3A11 clone	3C11 clone
D8S1179	13–14–15–16–17	13–14–15–16–17	13–14–15–16–17
D21S11	26–30.2	26–30.2	26–30.2
D7S820	9.1–10.1–11.2	9.1–10.1–11.2	9.1–10.1–11.2
CSF1PO	11–14–16	11–14–16	11–14–16
D3S1358	14–15	14–15	14–15
TH01	7–8–9.3	7–8–9.3	7–8–9.3
D13S317	8–10	8–10	8–10
D16S539	11–12–13	11–12–13	11–12–13
D2S1338	17–24–25	17–24–25	17–23–24
D19S433	13–14	13–14	12–13–14
vWA	16–20–21	16––19–22	16–20–21
TPOX	8–10–11	8–10–11	8–10–11
D18S51	14–15–16	15–16	15–16
Amelogenin	X	X	X
D5S818	12–13	12–13	12–13–16.1
FGA	20–21–25–26	20–21–25–26	20–21–24–26

Loci were analyzed using the multiplex-PCR-based Identifier amplification kit (Applied Biosystems), comprising 15 autosomal STR Loci and the sex-chromosome marker amelogenin.

on androgen-independent PC3 cell growth (Fig. 3B). Growth of LNCaP cells was increased through a daily treatment with 10^{-9} M DHT for 3 days to serve as a control.

IGR-CaP1 cells do not express AR or PSA but express other prostate-specific markers

IGR-CaP1 cells did not express androgen receptor protein (AR) or secretory prostate-specific antigen (PSA) as shown by blot analyses (Fig. 3C). The LNCaP and MDA-PCa-2b cells which both express these two prostate markers were used as positive controls while the PC3 cells were used as a negative control. FACS analysis confirmed the absence of PSA and AR in IGR-CaP1 cells, as compared to LNCaP and PC3 cell lines (Supplementary Fig. 1A). Furthermore, in IGR-CaP1 cells neither AR nor PSA were detected at mRNA levels by quantitative RT-PCR analyses (Fig. 3D). AR protein and PSA expression in the IGR-CaP1 cell line contrasted with that observed in the original tumor. The absence of AR and PSA expression in the early passages of the IGR-CaP1 cell line (Supplementary Fig. 1B) suggests that cells expressing AR were lost during the *in vitro* cell culture establishment. It is established that in prostatic epithelium, AR protein and PSA expression is restricted to luminal secretory prostate cells and is not observed in

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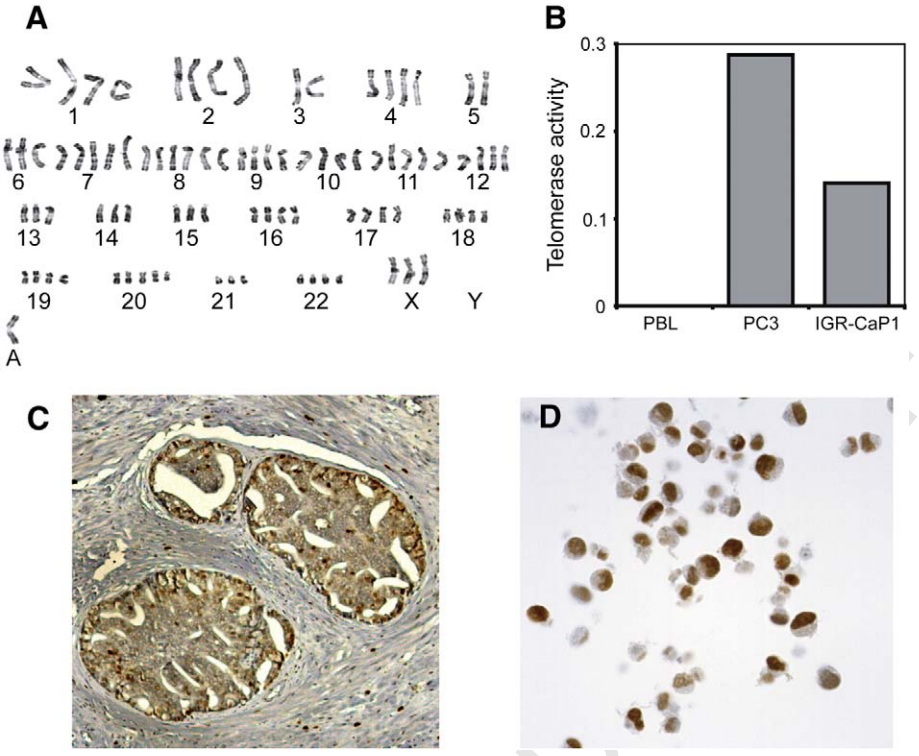

Fig. 2 – Malignancy features of the IGR-CaP1 cell line. (A) Karyotypic analysis of the IGR-CaP1 cell line using a conventional R-banding technique showing a complex tetraploid karyotype with 86-91 chromosomes. (B) Telomerase activity was quantified by quantitative PCR. Telomerase activity of IGR-CaP1 cells was compared to normal lymphocyte cells (PBL). PC3 cells were used as positive controls. (C–D) IHC showing high p53 expression in the initial prostatic tumor (C) and in the IGR-CaP1 cell line (D).

the basal layer cells of the epithelium, suggesting that IGR-CaP1 cells might correspond to basal epithelial prostate cancer cells.

Alpha-methylacyl-CoA-racemase (AMACR/ P504S) is a biomarker for prostate cancer that is strongly expressed in prostate cancer cell lines and tissues independently of androgen receptor expression [25,26]. As measured by FACS analysis, IGR-CaP1 cells expressed AMACR as also observed in LNCaP cells (Fig. 3E) and in PC3 cells (not shown). Like other prostate-specific markers, PSMA, the prostate-specific membrane antigen (also named FOLH1) [27] is expressed only in a small fraction (~2%) of IGR-CaP1 cells, as shown by flow cytometer analysis, compared to LNCaP cells (Fig. 3E). The low level of PSMA expression detected in *in vitro* culture conditions was in accordance with PSMA expression observed in orthotopic xenografts (Fig. 4B).

IGR-CaP1 cells reconstitute prostate adenocarcinoma in mice

To determine tumorigenicity of IGR-CaP1 cells in animals, cells were injected into male nude mice both subcutaneously and orthotopically. Subcutaneous injections of IGR-CaP1 cells resulted in the formation of palpable tumors within a week with 90% of mice (20/22) bearing tumors after 6–8 weeks (Fig. 4A). Orthotopic injection of IGR-CaP1 cells resulted in the formation of intraprostatic tumors (Fig. 4B). In all tumors, HES staining of sections revealed glandular differentiation with acini attesting the presence of adenocarcinoma. Immunohistochemical staining with Ki67 revealed a high proliferation index which was higher in the intraprostatic tumor than in subcutaneous xenografts, indicating more aggressive features in orthotopic tumors (Fig. 4A and B). All the neoplastic cells expressed cytokeratins, as shown with CK14 labelling (Fig. 4A), but none expressed chromogranin A indicating an epithelial phenotype (not shown). In addition, staining for both prostatic acid phosphatase (PAP) and prostate-specific membrane antigen (PSMA) ascertained the prostate origin of the tumor (Fig. 4A and B). Metastases were observed in animals with both intraprostatic and subcutaneous tumors and predominantly in the liver and lung (not shown). Androgen responsiveness was then evaluated *in vivo* by castrating animals ($n=5$), once IGR-CaP1 tumors attained a volume of approximately 300 mm^3. Castration resulted in a slight reduction of tumor progression although the difference was not statistically different (interaction p-value = 0.096) (Supplementary Fig. 2). This suggests that although the androgen receptor was not expressed in epithelial tumor cells, tumor progression was at least partially under androgen control, thus emphasizing the importance of the tumor microenvironment [28]. However, tumor progression in the castrated mice suggested that the epithelial tumor cells might possess specific properties that were contributing to the survival of tumor cells in response to androgen deprivation. Altogether, these data showed that tumors initiated by the IGR-CaP1 cell line recapitulate the characteristics of any initial human prostate cancer in mice.

IGR-CaP1 cells express high levels of basal epithelial prostate markers

To determine the phenotypes of IGR-CaP1 cells, we first examined the expression of the cytokeratin markers CK5, CK8, CK14 and CK18 by cytometry analysis (Fig. 5A). The cytokeratin expression profile of the luminal epithelial LNCaP cell line was used as a control. IGR-CaP1

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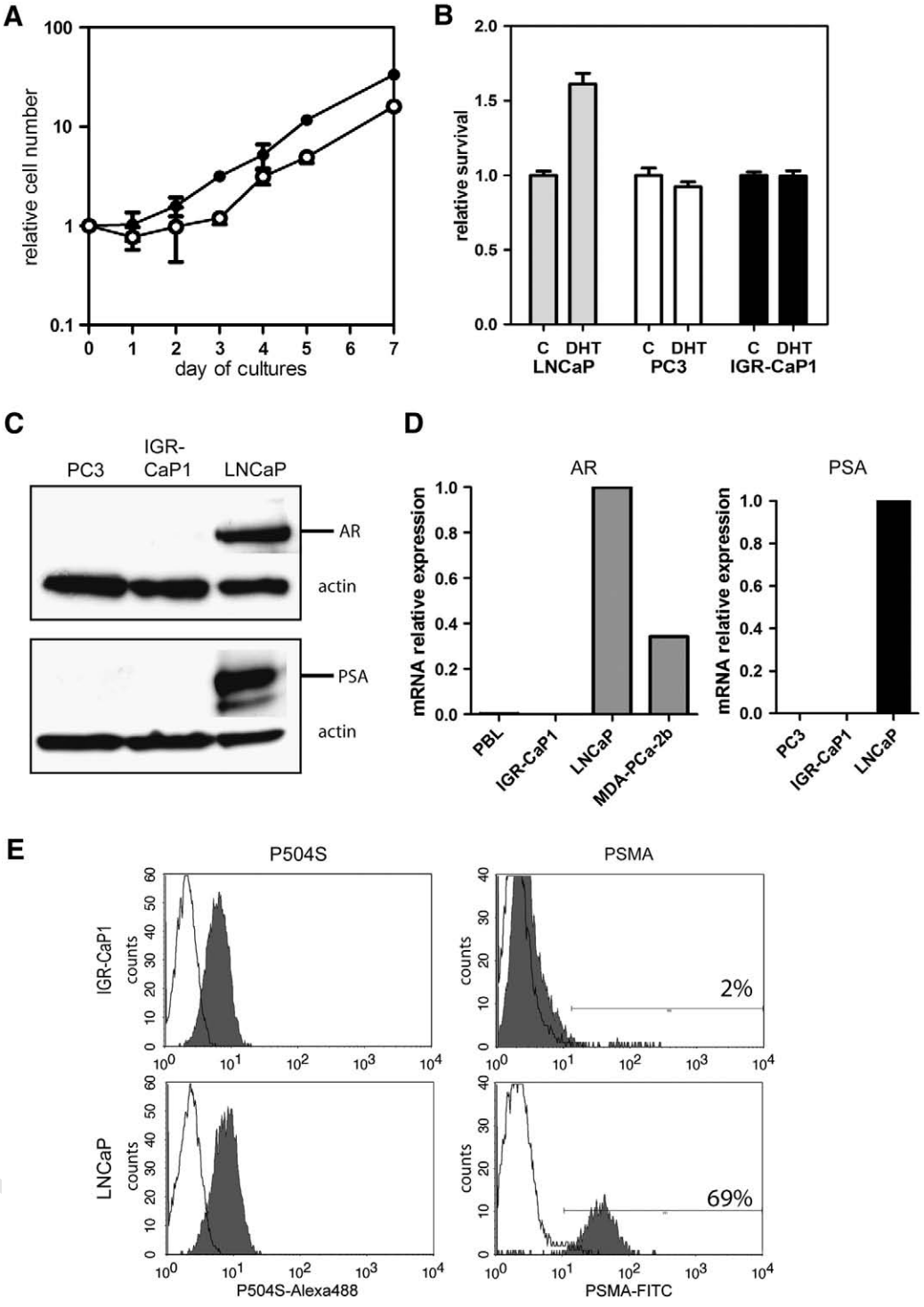

Fig. 3 – Cell growth, kinetics and expression of prostate-specific markers in IGR-CaP1 cells. (A) The growth rate of IGR-CaP1 cells at passage 28 was determined in culture medium supplemented with 10% FBS (●) or with 10% charcoal-stripped FBS (○). (B) The survival of IGR-CaP1, LNCaP and PC3 cells was determined after treatment of 10^{-9} M DHT for 72 h compared to no treatment (C) in medium supplemented with charcoal-stripped FBS. (C) Blot analysis showed no expression neither of AR nor of PSA in IGR-CaP1 cells compared to that found in PC3 and LNCaP cells respectively used as negative and positive controls. (D) The absence of AR and PSA gene expression was confirmed by quantitative RT-PCR analyses when compared with mRNA extracted from MDA-PCa-2b and from LNCaP cells as positive controls. (E) The expression level of the prostate markers P504S (AMACR) and PSMA was evaluated by flow cytometry. LNCaP cells were used as controls.

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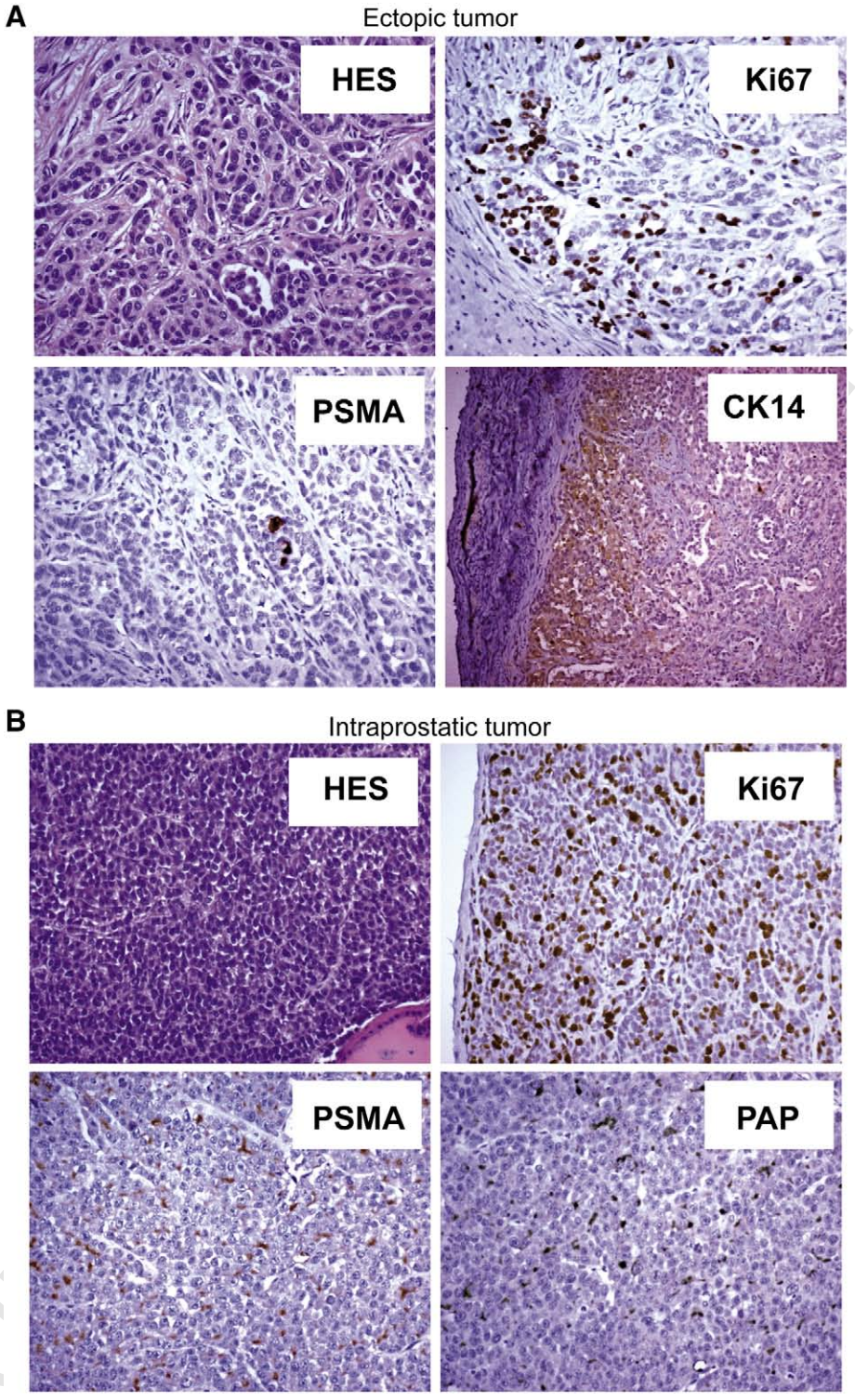

Fig. 4 – IGR-CaP1 cells reconstitute adenocarcinoma in mice. IGR-CaP1 cells were injected into male nude mice both subcutaneously (A) or intraprostatically (B). HES staining of tumor sections revealed glandular differentiation with acini confirming the presence of adenocarcinoma. CK14 attested the epithelial origin of the tumor. Ki67 staining revealed the proliferation index. Markers corresponding to prostatic acid phosphatase (PAP) and the prostate secreted membrane antigen (PSMA) confirmed the prostate origin of the tumor.

cells exhibited strong staining for CK5/8 and CK14 but no staining for CK8. Although adult prostate basal cells have been shown to express p63, we did not detect p63 expression in these cells (not shown). We detected low labelling for the luminal epithelial marker CK18 in IGR-CaP1 cells compared to that observed in LNCaP cells (93% CK18 fluorescence in LNCaP versus 34% in IGR-CaP1 cells). The absence of

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CK8 and the low level of CK18 expression indicated the absence of luminal differentiation in cultured IGR-CaP1 cells. Additionally, CK19 expression was detected in early passages of the IGR-CaP1 cell line, but disappeared during *in vitro* passaging and was not detectable in cells at passage 71 (not shown). The two clones 3A11 and 3C11 exhibited roughly the same cytokeratin expression profile. These results as a whole indicate that the IGR-CaP1 cell line displays a cytokeratin profile corresponding to basal epithelial cells. As prostate stem cells have been shown to exist in the basal cell compartment, these data suggested that a small subset of these cells expressing a broad spectrum cytokeratin profile might correspond to progenitor/stem cells.

IGR-CaP1 cells and clonally-derived clones show features of prostate cancer stem cells

Previous studies have suggested that putative prostate progenitor/stem cells are located in the basal layer and express markers of basal cells. As in the normal prostate, the putative stem cell population was shown to be enriched in CD44 and CD133 antigens [11], we first evaluated their expression levels in the IGR-CaP1 cell line by flow cytometry (Fig. 5B). Compared to LNCaP cells in which only a minor fraction expressed CD44, almost all the IGR-CaP1 cells expressed CD44 antigen. CD133 expression was not observed in LNCaP cells. In contrast, we detected two populations in IGR-CaP1 cells, one exhibiting high CD133 expression and the other was CD133-negative. CXCR4, a key molecule in the regulation of the migratory and metastatic properties of cancer cells, may be essential for the progression of the $CD133^+$ prostate cancer stem cells [15]. We found that a large subset of IGR-CaP1 cells expressed the CXCR4 molecule. In addition, we found that all the IGR-CaP1 cells were CD24 positive (Supplementary Fig. 3A). Real-time quantitative RT-PCR confirmed high expression of CD44 in the parental IGR-CaP1 cell line and in the clonally-derived clones 3A11 and 3C11 (mean Ct of 25.8) whereas CD44 gene expression was not detectable in LNCaP cells ($Ct > 40$) (not shown). High expression of CD133 and CXCR4 was also confirmed by RT-PCR performed at different passages (Fig. 5C). The combined biomarker expression after triple-labelling and flow cytometry analysis was evaluated and showed a high fraction of $CD44^+/CD133^+/CXCR4^+$ triple-labelled cells (13% of the population in the parental IGR-CaP1 cells , 23% and 34% in 3A11 and 3C11 clones respectively) (Supplementary Fig. 3B). Furthermore, as it has been postulated that anchorage-independent culture of tumor cells is a useful tool for enriching and characterizing stem cells, we next investigated the clonogenic capacity in a soft agar assay. The results showed that IGR-CaP1 cells were able to form spheroids (~200 μm) in serum-supplemented RPMI medium and HES staining showed glandular-like structures with a lumen (Fig. 5D). These findings suggest that IGR-CaP1 cells possess stem cell-like characteristics. We next investigated the ability of IGR-CaP1 cells to maintain their characteristics after xenografting into animals. Cells were xenografted subcutaneously into nude mice. Subsequently, after 2 months of tumor growth, tumor cells were dissociated by enzymatic digestion and plated in culture dishes in classic culture conditions. After 5 passages of *in vitro* culture, stem cell markers and basal epithelial markers were assessed by FACS analysis. The results in Fig. 5E show that even after xenografting into mice and solid tumor growth, the IGR-CaP1 cells retained the characteristics of prostate basal epithelial cells (CK5 and CK14) and the potential of CSC (CD44, CD133 and CXCR4). In particular, we still observed the same fraction of CD133 positive cells (~50% of the cells). Interestingly, CXCR4 expression was increased in these conditions (75% of CXCR4 positive cells). The small fraction of PSMA positive cells was also conserved. Thus, these results strengthened the value of the IGR-CaP1 model as a model system based on tumor stem cells originating from the basal epithelium.

IGR-CaP1 cells show a stem cell expression signature

As the $\alpha2\beta1^{hi}/CD133^+$ phenotype is the hallmark of normal prostate epithelial stem cells [11], α2β1-integrin staining of IGR-CaP1 and clonally-derived clones was performed (Fig. 6A). High expression of these phenotypic markers in the IGR-CaP1 cells and the two clones 3A11 and 3C11, suggested the occurrence of a large subset of cells exhibiting the characteristics of stem cells, and possibly corresponding to stem cells.

We then explored whether other signalling pathways involved in stemness could be activated in IGR-CaP1 cells. We investigated Oct4 which was previously reported to be expressed in pluripotent embryonic stem (ES) cells and in hTERT-immortalized HPE (HPET) cells [14,29]. Microarray analysis performed on IGR-CaP1 cells revealed Oct4 gene expression. By using an antibody that exclusively recognizes the Oct-4 (OCT4A) isoform-1, Western Blot analysis confirmed Oct4 expression in IGR-CaP1 cells, unlike that found in LNCaP and PC3 cells used as controls (Fig. 6B). Since the Hedgehog (HH) signalling pathway regulates key functions of stem/progenitor cell self-renewal [30,31], we used real-time RT-PCR to assess its expression in IGR-CaP1 cells compared to the LNCaP cell line. The SHH gene was highly expressed in IGR-CaP1 cells with concomitant expression of GLI1 and GLI2 target gene products. The HH receptor Patched protein PTCH, which normally blocks the pathway, was slightly downregulated in IGR-CaP1 cells (Fig. 6C). Similar results were obtained in the two clones 3A11 and 3C11, except for the GLI2 gene which exhibited higher expression in the 3A11 clone and lower expression in the 3C11 clone.

We then assessed the expression of two other proteins which have been implicated in stem-like cell populations. We observed 4-fold higher expression of NOTCH1 gene in IGR-CaP1 cells compared to LNCaP cells, consistent with previous results showing that Notch gene expression was restricted to basal epithelial cells [32]. In agreement with detection of the breast cancer resistance protein BCRP/ABCG2 in basal epithelial CD133+ prostate cells [33], IGR-CaP1 cells also expressed a high level of mRNA coding for ABCG2 gene (Fig. 6D). Similar results were obtained in the two clones 3A11 and 3C11.

Discussion

Herein, we describe the establishment and characterization of a new prostate cancer cell line named IGR-CaP1 obtained from a primary prostate cancer. Although cell lines from primary prostate carcinomas are among the most difficult to establish *in vitro*, we took advantage of the natural extracellular matrix (IGR-XC) we recently developed and already used successfully to obtain cell lines derived from human carcinomas of unknown primary [34], to obtain a new cell line derived from a patient with a clinically localized prostate cancer. The IGR-CaP1 cell line was cultured up to passage 50. Nine clones were derived by limiting dilution cloning and two of them,

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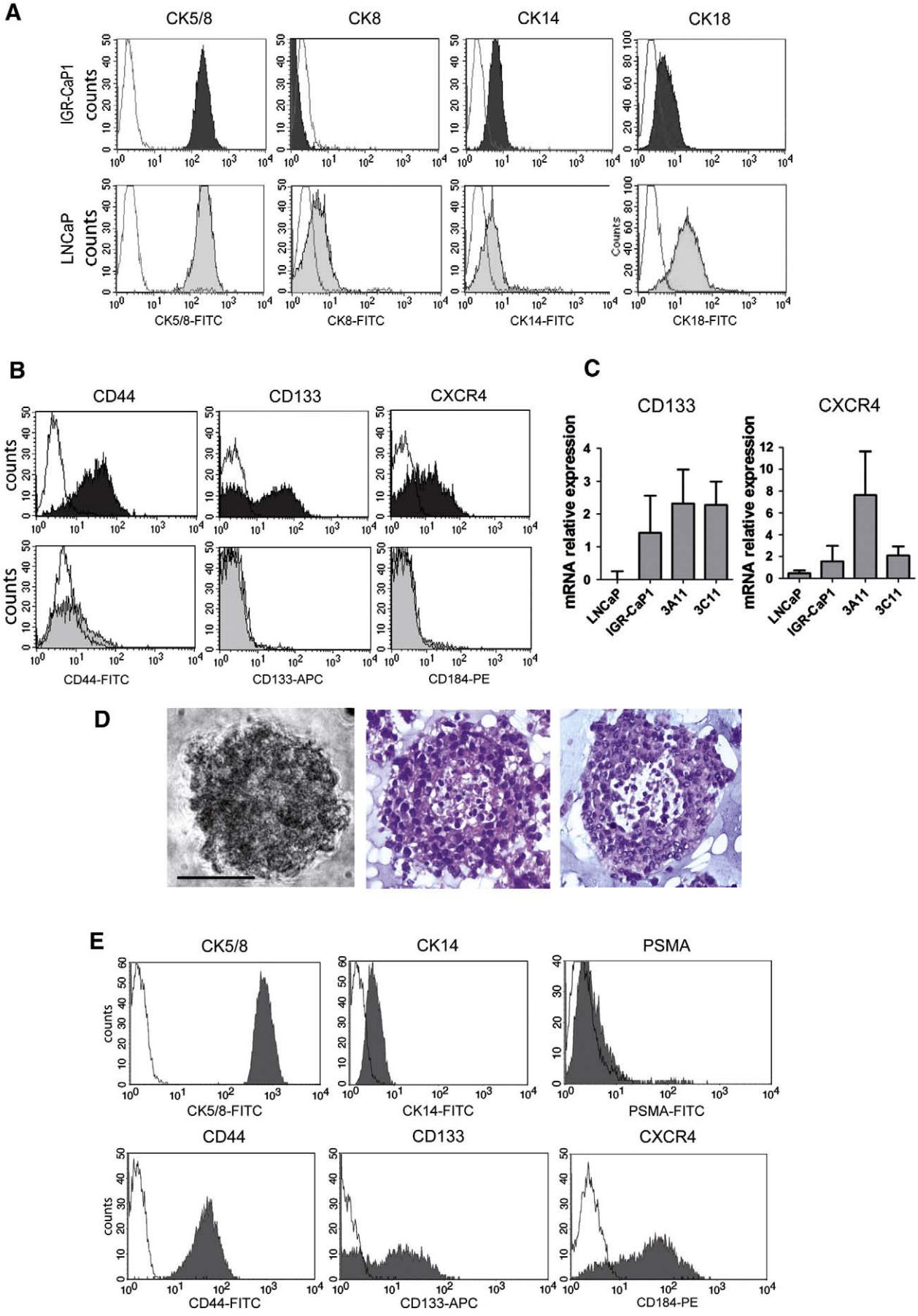

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3A11 and 3C11, were cultured up to passage 10. The complete identification of the new IGR-CaP1 cell line was achieved using STR profiling (Table 1).

The chromosome abnormalities confirmed malignancy in IGR-CaP1 cells which exhibited a tetraploid karyotype. Given the lack of prostate cancer models, telomerase-immortalized human prostate epithelial (HPET) cells provide useful models since they display prostate stem cell properties and also reconstitute the original prostate cancer specimen [14,15,31]. We showed that IGR-CaP1 cells spontaneously elicit high telomerase activity thus attesting the malignant potential of these cells. Undoubtedly, the endogenously hTERT-expressing IGR-CaP1 cells are more relevant to investigate the early steps of prostate oncogenesis because the key regulatory steps involved in telomerase activation in tumorigenesis may be missed in HPET cells. Somatic *Tp53* mutations in primary tumors are associated with prostate cancer progression and a propensity for metastasis [35,36]. In this respect, the missense mutation identified in the IGR-CaP1 cell line at position 126 of the Tp53 correlates with the high Tp53 protein expression found in both of these *in vitro* propagated cells and in the original tumor. It is possible that both properties, tetraploidy and mutated Tp53 expression, played a role in the establishment of the IGR-CaP1 cell line. Indeed, reports supported that tetraploid cells occur as an early step in tumor formation thus, conferring a survival advantage on tumor cells during the *in vitro* process [37]. Tetraploidy might have been maintained due to the presence of a mutated Tp53. Indeed, it has been shown that a "tetraploid checkpoint" is normally controlled by wild-type Tp53 to avoid the proliferation of tetraploid cells [38].

Interestingly, and consistent with the results obtained *in vitro*, the successful engraftment of IGR-CaP1 in nude mice plus their ability to metastasize to the liver and lung, enable us to propose IGR-CaP1 as an invaluable experimental model of PCa. Strikingly, preliminary data obtained 10 weeks after intra-tibia injections of the IGR-CaP1 cells into nude mice, imaged by high-resolution microCT scan, showed both osteoblastic and osteolysis lesions suggesting that these cells induced bone remodelling, as regularly observed in patient bone metastases (Supplementary Fig. 4).

IGR-CaP1 cells do not express AR nor the androgen-regulated gene PSA unlike the original tumor, suggesting a selective survival advantage of AR-negative cells and the loss of differentiated AR-positive cells during the early steps of *in vitro* tumor culture. Consistent with the lack of AR and PSA expression, the IGR-CaP1 cells express the basal epithelial cytokeratins, CK5 and CK14 and did not express the luminal CK8 markers. This is in full agreement with the working hypothesis of the occurrence of a small population of epithelial stem cells in the basal cell layer, giving rise to basal cells and intermediate transit-amplifying cells [31]. As we also detected CK18 expression, albeit at a much lower level, this strongly suggests that IGR-CaP1 cells mainly correspond to basal epithelial cells and that CK18 expression may be attributed to the existence of some intermediate transit-amplifying cells.

Considerable efforts are currently being expended to identify cells at the origin of prostate cancer. A growing body of literature supports that cancer lethality results from the hierarchical expansion of "cancer-initiating cells" or "cancer stem cells" (CSC), which function as stem-like cells to maintain malignant growth [39]. Several potential markers of CSCs have been used to identify putative CSCs in several solid cancers and can potentially be used to isolate and characterize CSCs. One of the major reported CSC markers is CD133 which is a marker for both human normal prostate stem cells and prostate CSC [11]. Other studies showed that putative prostatic stem cells with the $\alpha2\beta1^{hi}/CD44^{+}/CD133^{+}$ phenotype were able to reconstitute prostate-like acini in mice that expressed differentiated cell markers [12]. More recently, co-expression of CXCR4 was demonstrated in $CD133^{+}$ human prostatic epithelial cells immortalized with hTERT [15]. We showed that IGR-CaP1 cells exhibit high expression of cancer stem cell CD44, CD133 markers and CXCR4, and exhibit notably a high proportion (47%) of CD133-expressing cells thus clearly identifying two populations of CD133-positive and CD133-negative cells. Two sub-populations were also observed based on the wide difference in $\alpha2\beta1$-integrin labelling. Therefore, as multiple populations with CSC characteristics may co-exist either in the same cell line or in the same tumor, this strongly suggests that our cell line corresponds to CSC-derived cancer progenitor cells capable of giving rise to clonal expansion. Such an assessment is fully consistent with the conclusions of Campbell and Polyak, as well as Adams and Strasser [40,41] suggesting that although CSCs are a special subset of tumor cells, they still constitute a heterogeneous population with different biological properties.

There are several other signalling pathways including the HH and NOTCH pathways that are so far reported to be involved in the maintenance of stem or progenitor cells of many adult tissues, and also shown to operate in human PCa progression [42–48]. As a matter of fact, Sonic Hedgehog pathway (HH-GLI) activation and high expression of the NOTCH-1 gene suggest that this signalling pathway could be involved in the maintenance of the "CSC-like" population within the IGR-CaP1 cells. Oct-4 expression which was reported in HPET cells [14,16] and seems to play a crucial role in resistance to chemotherapy in lung cancer CD133+ cells [49], is also detected in IGR-CaP1 cells. It is noteworthy that high expression of the BCRP/ABCG2 gene provides an additional stem cell marker in the IGR-CaP1 cells. Taken together, this further suggests that the self-renewal genes including HH, Notch-1, Oct4 and ABCG2, may be implicated in the self-renewal properties of IGR-CaP1 cells.

In conclusion, the IGR-CaP1 cell line is a new model of prostate cancer derived from a primary tumor exhibiting high tumorigenic

Fig. 5 – Basal-type cytokeratin profile and cancer stem cell marker expression. (A) Cytokeratin marker expression was measured in IGR-CaP1 cells by FACS. LNCaP cells were used as controls. (B) The expression level of the stem cell markers CD44, CD133 and CXCR4 (also named CD184) was evaluated by flow cytometry. LNCaP cells were used as controls. (C) CD133 and CXCR4 gene expression levels were measured by QRT-PCR in IGR-CaP1 cells and in the clones 3A11 and 3C11 using a range of passages for the IGR-CaP1 cells (P26, P31, P48 and P64), P7, P8 and P10 for the 3A11 clone and P6, P7 and P9 for the 3C11 clone. Two different samples were used for the control LNCaP cell line. Results are expressed as means ± SD and compared to the LNCaP cells as the reference. (D) Anchorage-independent growth of IGR-CaP1 cells in soft-agar assay showing spheroids grown in RPMI medium. Representative HES-stained section. Scale-bar: 100 μm (E) Re-establishment of the IGR-CaP1 cell line after xenografting into nude mice. Basal cytokeratin profiles, stem cell markers and PSMA expression after engraftment in animals and re-establishment *in vitro* of IGR-CaP1 cells (P5). Results are representative of what was obtained in the 4 newly established cells after mouse engraftment.

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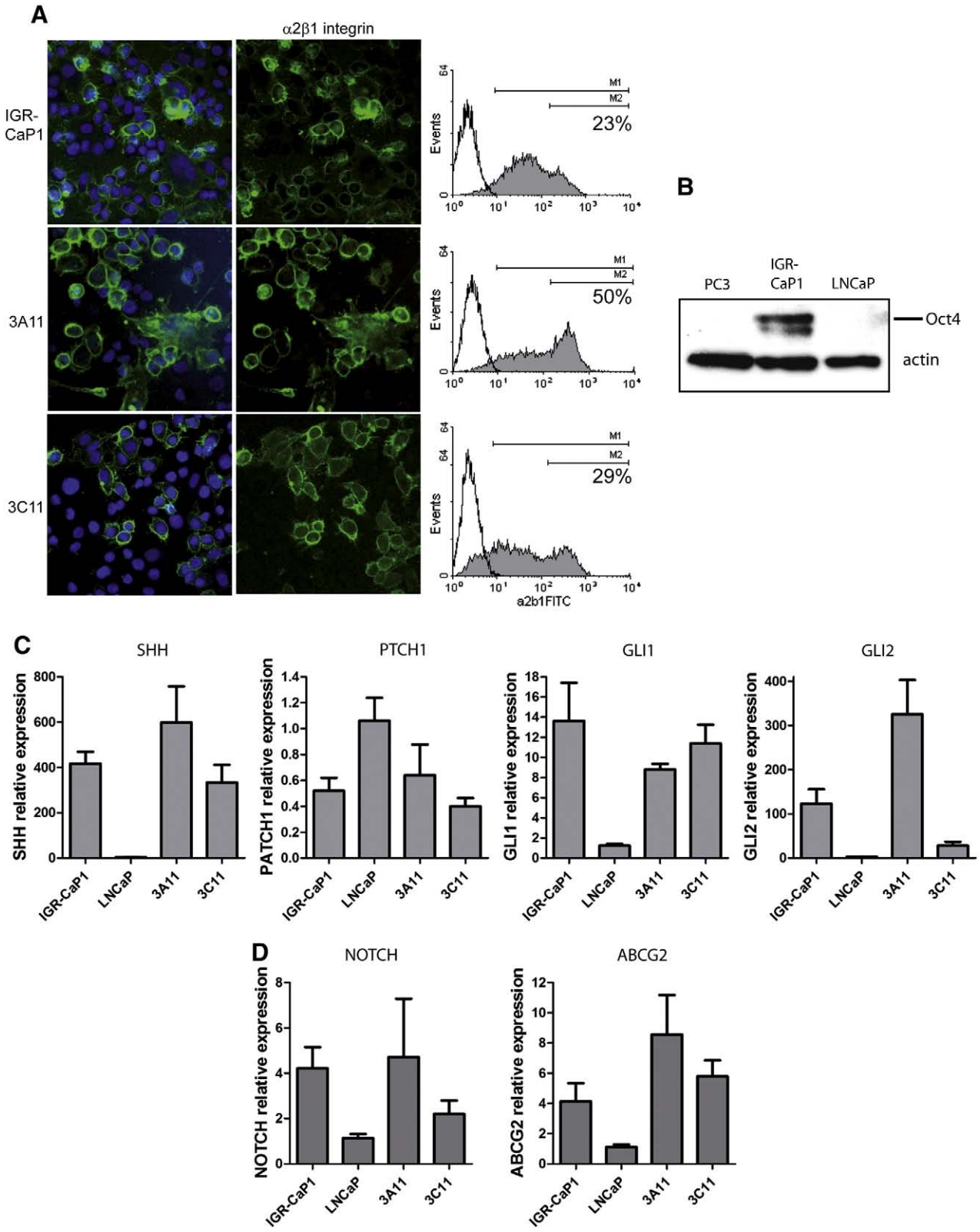

Fig. 6 – Stem cell signalling pathways in IGR-CaP1 cells. (A) α2β1-integrin was measured by immunofluorescence and by FACS in IGR-CaP1 cells and in the 3A11 and 3C11 clones, using anti-VLA-2 antibody. The nuclei were counterstained with Dapi. The fraction of α2β1integrin^{high}-expressing cells was estimated with the M2 marker (B) Oct4 expression was evaluated by blot analysis in IGR-CaP1 cell extracts and compared with that found in PC3 and LNCaP cells. (C and D) QRT-PCR was used to determine the expression level of markers identified as regulators of normal prostate stem/progenitor cells. Multiple passage levels were used as in Fig. 5C. Results are expressed as means ± SEM and compared to the LNCaP cells used as controls.

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properties *in vivo*. Spontaneously immortalized, these mutated Tp53 tetraploid cells express hTERT and high-level expression of stem cell markers (CD44, CD133, α2β1-integrin). Additionally, the IGR-CaP1 cells possess additional CSC characteristics, namely a 3D sphere-forming ability and a renewal capacity by maintaining their CSC potential after xenografting into mice. Thus, the IGR-CaP1 cell line derived from a primary tumor represents an experimental model of an aggressive prostate tumor with stem cell properties. Moreover, the stem cell signature provides an invaluable tool for investigating the mechanisms of resistance to chemotherapy and radiation. It could be used to study new therapeutic strategies both *in vitro* and *in vivo*, namely new therapeutic strategies targeting the stem cell properties of cancer cells.

Supplementary materials related to this article can be found online at doi:10.1016/j.yexcr.2010.10.012.

Disclosure statement

This new IGR-CaP1 cell line was the subject of a European patent pending entitled "Prostate cancer cell lines and their use in screening method" deposed on the April 14th, 2009. Biological material has been deposited at the Pasteur Institut (Paris) (CNCM I-4126). The patented material will be available under a Material Transfer Agreement for research use.

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