Research Article

Store-Operated Ca\(^{2+}\) Entry Does Not Control Proliferation in Primary Cultures of Human Metastatic Renal Cellular Carcinoma

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Store-operated Ca\(^{2+}\) entry (SOCE) is activated following depletion of the inositol-1,4,5-trisphosphate (InsP\(_3\))-sensitive Ca\(^{2+}\) pool to regulate proliferation in immortalized cell lines established from either primary or metastatic lesions. The molecular nature of SOCE may involve both Stim1, which senses Ca\(^{2+}\) levels within the endoplasmic reticulum (ER) Ca\(^{2+}\) reservoir, and a number of a Ca\(^{2+}\)-permeable channels on the plasma membrane, including Orai1, Orai3, and members of the canonical transient receptor (TRPC1–7) family of ion channels. The present study was undertaken to assess whether SOCE is expressed and controls proliferation in primary cultures isolated from secondary lesions of heavily pretreated metastatic renal cell carcinoma (mRCC) patients. SOCE was induced following pharmacological depletion of the ER Ca\(^{2+}\) store, but not by InsP\(_3\)-dependent Ca\(^{2+}\) release. Metastatic RCC cells express Stim1-2, Orai-3, and TRPC1–7 transcripts and proteins. In these cells, SOCE was insensitive to BTP-2, 10 \(\mu\text{M} \) Gd\(^{3+}\) and Pyr6, while it was inhibited by 100 \(\mu\text{M} \) Gd\(^{3+}\), 2-APB, and carboxyamidotriazole (CAI). Neither Gd\(^{3+}\) nor 2-APB or CAI impaired mRCC cell proliferation. Consistently, no detectable Ca\(^{2+}\) signal was elicited by growth factor stimulation. Therefore, a functional SOCE is expressed but does not control proliferation of mRCC cells isolated from patients resistant to multikinase inhibitors.
1. Introduction

Renal cell carcinoma (RCC) is by far the most common kidney cancer, accounting for 2%-3% of all adult malignancies and representing the 7th most common cancer in men and the 9th most common cancer in women [1]. The only treatment for patients with localized RCC is radical surgical resection of the tumour; however, 20–30% of patients present with synchronous metastases at diagnosis, and up to 30–35% of more patients, initially radically resected, will eventually develop metastases over time [2]. Targeting the intricate vascular network which develops around the primary tumour and is the gateway for metastasis dissemination has emerged as the most active treatment strategy to fight metastatic RCC (mRCC) [3–7]. In the past 7-8 years, the development and registration of molecularly targeted agents, inhibiting either the vascular endothelial growth factor (VEGF)/VEGF(Rs) pathway or mammalian target of rapamycin (mTOR), have dramatically improved the prognosis of mRCC patients [6, 7]. However, despite this abundance of active reagents, progression-free survival (PSF) for the majority of these patients rarely exceeds eleven months [6]. This is due to the ultimate development of drug resistance in the majority of patients initially responsive to treatment (3-4) and to the presence of so-called primary refractory patients (i.e., those who do not respond from the very beginning) [8]. There is, therefore, an unmet demand for discovering alternative molecular targets to achieve the complete regression of spreading metastases in both resistant and refractory mRCC patients, as well as in those experiencing treatment-related adverse events.

Store-operated Ca\(^{2+}\) entry (SOCE) is a ubiquitous mechanism which plays a key role in several key steps to malignant transformation, such as cell proliferation, migration, and metastasization [9–14]. A variety of molecular mechanisms have been proposed to sustain SOCE [4, 15]. Both G-protein coupled receptors (GPCRs) and tyrosine kinase receptors (TKRs) engage distinct isoforms of phospholipase C (PLC), that is, PLC\(\beta\) and PLC\(\gamma\) respectively, to cleave phosphatidylinositol 4,5-bisphosphate, into inositol-1,4,5-triphosphate (InsP\(_3\)) and diacylglycerol (DAG). InsP\(_3\) induces a massive Ca\(^{2+}\) release from the endoplasmic reticulum (ER), the most abundant intracellular Ca\(^{2+}\) reservoir, by activating the so-called InsP\(_3\) receptors (InsP\(_3\)Rs). The consequent fall in intraluminal Ca\(^{2+}\) levels is detected by the ER Ca\(^{2+}\) sensor, Stim1 [4, 16]. Once activated, Stim1 proteins aggregate and migrate towards subplasmalemmal puncta, where they tether to and gate the Ca\(^{2+}\)-selective channel Orai [4, 13]. Stim1 and Orai-mediated SOCE control cell cycle progression and metastatization in several human malignancies, including those of glia [17], liver [18, 19], breast, and uterine cervix [20]. Conversely, no functional role has so far been established for Stim1 and Orai paralogues, that is, Stim2 and Orai2, in carcinogenesis, while Orai3 is recruited by Stim1 to activate SOCE and cell proliferation in some oestrogen receptor-negative human breast cancer lines [21]. Alternatively, Stim1 might trigger SOCE by conveying the ER Ca\(^{2+}\) load to nonelective Ca\(^{2+}\)-permeable pathways, such as those belonging to the canonical transient receptor (TRPC1-7) family of ion channels [15]. Stim1 may bind to and activate TRPC1, TRPC4, and TRPC5; in addition, Stim1 causes TRPC3 and TRPC6 to mediate SOCE by mediating the heteromultimerization of TRPC3 with TRPC1 and of TRPC6 with TRPC5 [15]. TRPC1 and TRPC6 have convincingly linked to SOCE in a growing number of human tumours, including breast carcinoma [22], glioblastoma multiforme (GBM) [23, 24], and hepatoma [18, 19]. These results put forward SOCE as an alternative target to devise novel antimetastatic treatments [10, 12, 14, 25]. With some notable exceptions [17], however, the physiological function of SOCE has been characterized in immortalized cell lines established from either primary or metastatic lesions that do not accurately recapitulate the heterogeneity and complexity of human neoplasms. This issue is particularly relevant when seeking alternative strategies to fully eradicate metastases by inhibiting SOCE. Accordingly, earlier work showed that oncogenesis reduces the need for Ca\(^{2+}\) entry to initiate DNA synthesis and cell replication, a phenomenon known as “habituation” to extracellular Ca\(^{2+}\) [26, 27].

We have recently demonstrated that Stim1, Orai, and TRPC1 mediate SOCE and control proliferation in circulating endothelial progenitor cells (EPCs) isolated from untreated mRCC patients [10]. The present study aimed to assess whether a functional store-dependent Ca\(^{2+}\) inflow is present and drives cell growth in a different cellular setting related to this neoplasm, that is, primary cultures of mRCC cells isolated from subjects resistant to multikinase inhibitors.

2. Materials and Methods

2.1. Tumour Samples. Tumour samples were collected from 4 patients affected by metastatic renal carcinoma (mRCC) who have undergone surgical intervention or biopsy to remove metastasis (Table 1), in accordance with a protocol approved by the local Ethics Committee and after signing informed consent. We reasoned that it would be of particular interest to investigate also cells derived from primary tumour to define whether metastatic cells presented peculiar features; however, during all the observation time, we never had the availability of patients undergoing simultaneous resection of both primary tumour and metastases. So, we decided to focus this first study only on the characterization of metastatic cells. Surgical materials not required for histopathologic diagnosis were placed in sterile tubes containing RPMI 1640 supplemented with 10% heat-inactivated FBS, 200 U/mL penicillin, and 200 μg/mL streptomycin (all from Life Technologies Inc., Paisley, UK).

2.2. Establishment of Primary Cell Cultures from Surgical Samples. Tumour samples were treated with 0.1% collagenase for half an hour and then processed with the gentle MACS Dissociator (Miltenyi Biotec, Germany), according to the manufacturer’s instructions. Tumour cells were filtered (Miltenyi Biotec, Germany) to remove clusters and then collected by centrifugation at 1000 rpm for 10 minutes, checked for viability with trypan blue dye exclusion, resuspended at a concentration of 0.5–1 × 10^6 cells/mL of CellGro SCGM (Cell Genix, Freiburg, Germany), supplemented with 20%...
Table 1: Clinical characteristics of renal cell carcinoma patients involved in this study.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Day of birth</th>
<th>Year of diagnosis</th>
<th>Histology</th>
<th>Stage¹ at diagnosis</th>
<th>Surgery for localized disease</th>
<th>Systemic treatments received</th>
</tr>
</thead>
<tbody>
<tr>
<td>LA</td>
<td>M</td>
<td>14/11/1944</td>
<td>1995</td>
<td>Clear cell RCC</td>
<td>pT2, Nx, M0</td>
<td>Yes, radical nephrectomy</td>
<td>IL-2, Sorafenib, Sunitinib, Everolimus, Experimental vaccine</td>
</tr>
<tr>
<td>DPV</td>
<td>M</td>
<td>21/05/1953</td>
<td>2010</td>
<td>Bellini's duct RCC</td>
<td>pT2, N2, M1</td>
<td>Yes, Radical nephrectomy</td>
<td>Temsirolimus, Sorafenib, Sunitinib, Cytotoxic chemotherapy, Experimental vaccine</td>
</tr>
<tr>
<td>LG</td>
<td>F</td>
<td>25/12/1948</td>
<td>2005</td>
<td>Clear cell RCC</td>
<td>pT2, N0, M0</td>
<td>Yes, Partial nephrectomy</td>
<td>Cytotoxic chemotherapy, Sunitinib, Everolimus, Interferon-α, Experimental vaccine</td>
</tr>
<tr>
<td>GG</td>
<td>M</td>
<td>20/10/1959</td>
<td>2009</td>
<td>Papillary type I RCC</td>
<td>pT3b, N0, M1</td>
<td>Yes, radical nephrectomy</td>
<td>Sunitinib, Sorafenib, Everolimus, Cytotoxic chemotherapy, Experimental vaccine</td>
</tr>
</tbody>
</table>

¹Stage is indicated according to the 2002 TNM staging system.

FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (complete medium) (all from Life Technologies Inc.), and cultured in 25 cm² tissue flasks (Corning, Stone Staffordshire, England) at 37°C and 5% CO₂. Viable tumour cells attached to the flask within 12-24 hours. At the first medium change, rather than discarding medium containing unattached cells that may grow and provide a backup culture, we put these into a fresh flask. Cultures at 75% to 100% confluence were selected for subculture by trypsinization with 0.25% trypsin and 0.02% EDTA (Life Technologies Inc.) in a calcium/magnesium-free balanced solution. The culture medium was changed twice a week and cellular homogeneity evaluated microscopically every 24-48 hours.

To confirm the neoplastic origin of cultured cells, at least three cytopsins were performed using 10⁵ cultured cells/cytopsin obtained after 3-5 passages, for morphological and immunocytochemical analysis. Cells were fixed in alcohol 95%; one slide was stained with haematoxylin-eosin to identify malignant cells on the basis of cytomorphology. To distinguish tumour from hyperplastic mesothelial cells, the other slides were tested with monoclonal antibodies against cytokeratin CAM 5.2 (Dako, Glostrup, Denmark) and calretinin (Invitrogen), using indirect immunoenzymatic staining according to the manufacturers’ instructions. Tumour cells were assessed by a semiquantitative method as recently illustrated in [28]. In addition, since the great majority of metastatic clear cell RCC (by far the commonest histotype of kidney cancer) express CD10, cultured cells were also evaluated by cytofluorimetric analysis for the expression of this antigen. CD10 is usually not present on normal cells [29]. Evaluation of CD10 expression was performed by direct immunofluorescence, using phycoerythrin (PE)-anti CD10 specific monoclonal antibody (BD Pharmingen, Bioscience, Mountain View, CA), according to previously reported methods [30]. For functional experiments, tumour cells were thawed and plated at the concentration of 10-20 × 10⁵/mL and evaluated after 3-4 days when they reached the optimal confluency. In the proliferation assays, results are expressed as average number of cells (±SE) under each condition; the cells were obtained from all four patients. Differences were assessed by Student’s t-test for unpaired values. All statistical tests were carried out with GraphPad Prism 4.

2.3. Isolation and Culturing of Endothelial Progenitor Cells.

Since a number of drugs and agonists failed to affect intracellular Ca²⁺ dynamics in mRCC cells, we sought to verify their efficacy in another cellular setting, such as circulating EPCs isolated from healthy donors. We have recently shown that Ca²⁺ signaling is a key to EPC activation [9–11, 31, 32], so that they are suitable to serve as positive control for many substances commonly utilized to study subcellular Ca²⁺ movements [3, 25]. Blood samples (40 mL) were obtained from healthy human volunteers aged from 22 to 28 years old. The Institutional Review Board at “Istituto di Ricovero e Cura a Carattere Scientifico Policlinico San Matteo Foundation” in Pavia approved all protocols and specifically approved this study. Written informed consent was obtained according to the Declaration of Helsinki. We focused on the so-called endothelial colony forming cells (ECFCs) [33], a subgroup of EPCs which are found in the CD34⁺ CD45⁻ fraction.
of circulating mononuclear cells and exhibit robust proliferative potential and form capillary-like structures in vitro [4, 33]. To isolate ECFCs, mononuclear cells (MNCs) were separated from peripheral blood (PB) by density gradient centrifugation on lymphocyte separation medium for 30 min at 400 g and washed twice in EBM-2 with 2% FCS. A median of 36 × 10⁶ MNCs (range 18–66) was plated on collagen-coated culture dishes (BD Biosciences) in the presence of the endothelial cell growth medium EGM-2 MV Bullet Kit (Lonza) containing endothelial basal medium (EBM-2), 5% foetal bovine serum, recombinant human (rh) EGF, rhVEGF, rhFGF-B, rhIGF-1, ascorbic acid, and heparin and maintained at 37°C in 5% CO₂ and humidified atmosphere. Discard of nonadherent cells was performed after 2 days; thereafter, medium was changed three times a week. The outgrowth of endothelial cells from adherent MNCs was characterized by the formation of a cluster of cobblestone-appearing cells [11]. That ECFC-derived colonies belonged to endothelial lineage was confirmed as described in [10, 11].

2.4. Solutions. Physiological salt solution (PSS) had the following composition (in mM): 150 NaCl, 6 KCl, 1.5 CaCl₂, 1 MgCl₂, 10 Glucose, and 10 Hepes. In Ca²⁺-free solution (0Ca²⁺), Ca²⁺ was substituted with 2 mM NaCl, and 0.5 mM EGTA was added. Solutions were titrated to pH 7.4 with NaOH. The solution was then titrated to pH 7.4 with KOH. The osmolality of the extracellular solution was 338 mmol/kg, as measured with an osmometer (Wescor 5500, Logan, UT).

2.5. [Ca²⁺], Measurements and Statistics of Ca²⁺ Signals. mRCC cells were loaded with 4 μM fura-2 acetoxyethyl ester (fura-2/AM; 1 mM stock in dimethyl sulfoxide) in PSS for 1 hour at room temperature. After washing in PSS, the coverslip was fixed to the bottom of a Petri dish and the cells were observed by an upright epifluorescence Axioscop microscope (Carl Zeiss, Oberkochen, Germany), usually equipped with a Zeiss x40 Achroplan objective (water-immersion, 2.0 mm working distance, 0.9 numerical aperture). The cells were excited alternately at 340 and 380 nm, and the emitted light was detected at 510 nm. A first neutral density filter (1 or 0.3 optical density) reduced the overall intensity of the excitation light and a second neutral density filter (optical density = 0.3) was coupled to the 380 nm filter to approach the intensity of the 340 nm light. A round diaphragm was used to increase the contrast. The excitation filters were mounted on a filter wheel (Lambda 10, Sutter Instrument, Novato, CA, USA). Custom software, working in the LINUX environment, was used to drive the camera (Extended-ISIS Camera, Photonic Science, Millham, UK) and the filter wheel and to measure and plot on-line the fluorescence from 10 up to 100 rectangular “regions of interest” (ROI). Each ROI was identified by a number. Since cell borders were not clearly identifiable, a ROI may not include the whole cell or may include part of an adjacent cell. Adjacent ROIs never superimposed. [Ca²⁺], was monitored by measuring, for each ROI, the ratio of the mean fluorescence emitted at 510 nm when exciting alternatively at 340 and 380 nm (shortly termed “ratio”). An increase in [Ca²⁺], causes an increase in the ratio [34]. Ratio measurements were performed and plotted on-line every 3 s. The experiments were performed at room temperature (22°C). All the data have been collected from mRCC cells isolated from all four patients and from EPCs harvested from three different healthy donors. The amplitude of the peak Ca²⁺ response was measured as the difference between the ratio at the peak (either of intracellular Ca²⁺ mobilization in 0Ca²⁺ or of Ca²⁺ entry occurring upon Ca²⁺ restoration to the bath) and the mean ratio of 1 min baseline before the peak. Pooled data are given as mean ± SE and statistical significance (P < 0.05) was evaluated by Student’s t-test for unpaired observations.

2.6. RNA Isolation and Real Time RT-PCR (qRT-PCR). Total RNA was extracted from mRCC cells derived from all the four established cultures by using the QIAzol Lysis Reagent (QIAGEN, Italy). Single cDNA was synthesized from RNA (1 μg) using random hexamers and M-MLV Reverse Transcriptase (Invitrogen S.R.L., Italy). Reverse transcription was always performed in the presence or absence (negative control) of the reverse transcriptase enzyme. qRT-PCR was performed in triplicate using 1 μg cDNA and specific primers (intron-spanning primers) for Stim1-2, Orai-3, TRPC-7, and InsP3R3-3, as previously described [10, 11] (Tables 2, 3, and 4). Briefly, GoTelq qPCR Mastermix (Promega, Italy) was used according to the manufacturer instruction and qRT-PCR was performed using Rotor Gene 6000 (Corbett, Concord, NSW, Australia). The conditions were as follows: initial denaturation at 95°C for 5 min; 40 cycles of denaturation at 95°C for 30 sec; annealing at 58°C for 30 sec, and elongation at 72°C for 40 sec. The qRT-PCR reactions were normalized using β-actin as housekeeping gene. Melting curves were generated to detect the melting temperatures of specific products immediately after the PCR run. The triplicate threshold cycles (Ct) values for each sample were averaged resulting in mean Ct values for both the gene of interest and the housekeeping gene β-actin. The gene Ct values were then normalized to the housekeeping gene by taking the difference: ΔCt = Ct[gene] – Ct[β-actin], with high ΔCt values reflecting low mRNA expression levels. The sequences of the bands were checked by using the Big dye terminator cycle sequencing kit (Applied Biosystem, PE, USA). PCR products were also separated with agarose gel electrophoresis, stained with ethidium bromide, and acquired with the Image Master VDS (Amersham Biosciences Europe, Italy). The molecular weight of the PCR products was compared to the DNA molecular weight marker VIII (Roche Molecular Biochemicals, Italy).

2.7. Sample Preparation and Immunoblotting. mRCC were homogenized by using a Dounce homogenizer in a solution containing: 250 mM sucrose, 1 mM EDTA, 10 mM Tris-HCl, pH 7.6, 0.1 mg/mL PMSF, 100 mM β-mercaptoethanol, and Protease Inhibitor Cocktail (P8340, Sigma, USA). The homogenates were solubilized in Laemmli buffer [32] and 30 μg proteins were separated on 10% SDS-polyacrylamide gel electrophoresis and transferred to the Hybond-P PVDF Membrane (GE Healthcare, Italy) by electroelution.
Table 2: Primer sequences used for real time reverse transcription/polymerase chain reaction of Orai1–3 and Stim1-2.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences</th>
<th>Size (bp)</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orai1</td>
<td>Forward</td>
<td>5'-AGTTAATTCGTGGTGGATGAG-3'</td>
<td>257</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>5'-ATCGAGGGCTGTAGATGAG-3'</td>
<td></td>
</tr>
<tr>
<td>Orai2</td>
<td>Forward</td>
<td>5'-CCATAAGGCGATTAGC-3'</td>
<td>334</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-CAGGTGTGATGATGATG-3'</td>
<td></td>
</tr>
<tr>
<td>Orai3</td>
<td>Forward</td>
<td>5'-CCAAGCTCAAGGTGCT-3'</td>
<td>159</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>5'-CAAGAGGCTGAGCCGACCA-3'</td>
<td></td>
</tr>
<tr>
<td>Stim1</td>
<td>Forward</td>
<td>5'-CCTCAGTATAGGAGACCTTT-3'</td>
<td>347</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>5'-TCCTGAAGGTCATGCAGACT-3'</td>
<td></td>
</tr>
<tr>
<td>Stim2</td>
<td>Forward</td>
<td>5'-AAACACAGGATGCAGACAG-3'</td>
<td>186</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>5'-GGGAAGTGTCGTTCCTTTGA-3'</td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td></td>
<td></td>
<td></td>
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Table 3: Primer sequences used for real time reverse transcription/polymerase chain reaction.

<table>
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<th>Primer sequences</th>
<th>Size (bp)</th>
<th>Accession number</th>
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<td>TRPC1</td>
<td>Forward</td>
<td>5'-ATCTTACACTGGTGGCAGAA-3'</td>
<td>307</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>5'-AACAAAGCAAGAGGCTGTC-3'</td>
<td></td>
</tr>
<tr>
<td>TRPC3</td>
<td>Forward</td>
<td>5'-GGAGATCTGGAATCAGCAGA-3'</td>
<td>336</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-AACAAAGCAAGAGGCTGTC-3'</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Forward</td>
<td>5'-ACCTGGGACCTCTGCAAATA-3'</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-ACATGGTGGCACCAACAAAC-3'</td>
<td></td>
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<td></td>
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</tr>
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<td>TRPC4</td>
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<td>TRPC5</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>5'-AGACAGCATGGGAAACAGGA-3'</td>
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<tr>
<td>TRPC6</td>
<td>Forward</td>
<td>5'-AGCGTCTGGGGCCATGAA-3'</td>
<td>341</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>5'-AGCGTCTGGGGCCATGAA-3'</td>
<td></td>
</tr>
<tr>
<td>TRPC7</td>
<td>Forward</td>
<td>5'-CACTTGGGAACCTGCTGTA-3'</td>
<td>387</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-CATTCCAATCATGAGGCGCA-3'</td>
<td></td>
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blocking with Tris buffered saline (TBS) containing 3% BSA and 0.1% Tween (blocking solution), the membranes were incubated for 3 h at room temperature with the following affinity purified antibodies diluted 1:200 in the TBS and 0.1% Tween: anti-Stim1 (sc-166840), anti-Orai1 (sc-68895), anti-TRPC1 (sc-133076), anti-TRPC3/6/7 (sc-15056), and anti-IP3R-I/II/III (sc-377518) from Santa Cruz Biotechnology, anti-Orai3 (HPA015022) and anti-Stim2 (PRS4123) from Sigma-Aldrich (Italy), and anti β-actin rabbit antibody as control (Rockland Immunochemicals for Research, USA; code, 600-401-886). The membranes were washed and incubated for 1 h with peroxidase-conjugated mouse, rabbit, or goat IgG (1:120000 in blocking solution), from Dakocytomation (P0260), Chemicon (API32P), and Santa Cruz (sc-2354), respectively. The bands were detected with the ECL Select western blotting detection system (GE Healthcare Europe GmbH, Italy). Prestained molecular weight markers (SDS7B2, Sigma, Italy) were used to estimate the molecular weight of the bands. Control experiments were performed by using the antibody preadsorbed with a 20-fold molar excess of the immunizing peptide or by incubating the blots with nonimmune serum.

2.8. Protein Content. Protein contents of all the samples were determined by the Bradford’s method using bovine serum albumin (BSA) as standard [35].

2.9. Chemicals. EBM and EGM-2 were purchased from Clonetics (Cell System, St. Katharinen, Germany). Fura-2/AM was obtained from Molecular Probes (Molecular Probes Europe BV, Leiden, the Netherlands). N-(4-[3,5-bis(trifluoromethyl)-1H-pyrazo 1-yl]phenyl)-4-methyl-1,2,3-thiadiazole-5-carboxamide (BTP-2) was purchased from Calbiochem (La Jolla, CA, USA). CAI was a gift from the Drug Synthesis and Chemistry Branch, National Cancer Institute (Bethesda, MD). Pyr6 has been synthesized as described in [36]. All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA).
Table 4: Primer sequences used for real time reverse transcription/polymerase chain reaction of InsP₃ receptors.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences</th>
<th>Size (bp)</th>
<th>Accession number</th>
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<tbody>
<tr>
<td>InsP₃R1</td>
<td>Forward</td>
<td>5'-TCAACAAACTGCACCACGCT-3'</td>
<td>180</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>5'-CTCTCATGGGATTCCTTCTCC-3'</td>
<td></td>
</tr>
<tr>
<td>InsP₃R2</td>
<td>Forward</td>
<td>5'-ACCTTGGGTTAGTGGATGA-3'</td>
<td>158</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-CCTTGTTTGCTGTGGTCTTG-3'</td>
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<tr>
<td>InsP₃R3</td>
<td>Forward</td>
<td>5'-TGGCTTCATGCACCTTTTG-3'</td>
<td>173</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>5'-TGTCCTGCTTAGTCTGCTTG-3'</td>
<td></td>
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<tr>
<td>β-actin</td>
<td>Forward</td>
<td>Hs_ACTB_J_SG, QuantiTect Primer Assay QT00095431, Qiagen</td>
<td>146</td>
</tr>
</tbody>
</table>

3. Results

3.1. Store-Operated Ca²⁺ Entry Is Functionally Expressed in mRCC Cells. Primary cell cultures of mRCC were performed as described in Materials and Methods section and their morphology after staining with hematoxylin and eosin is illustrated in Figure 1(a). Immunofluorescence revealed that >95% of cells were positive for CD10 staining, thereby confirming that they belonged to the neoplastic phenotype (Figure 1(b)). Store-operated Ca²⁺ entry in primary cultures of mRCC cells was, thus, evaluated by exploiting the “Ca²⁺ add-back” protocol [10]. Fura-2-loaded cells were challenged with cyclopiazonic acid (CPA) and thapsigargin, two well-known inhibitors of the Sarco-Endoplasmic Reticulum Ca²⁺ ATPase (SERCA). These drugs prevent the pump from counterbalancing the passive Ca²⁺ leak from the stores to the cytosol, thereby leading to a massive drop in the ER Ca²⁺ content which signals the gating of store-operated Ca²⁺ channels on the plasma membrane [10]. As depicted in Figures 2(a) and 2(b), both CPA (10 μM) and thapsigargin (2 μM) elicited a robust SOCE in mRCC cells. Similarly, SOCE was activated by ionomycin (5 μM) (Figure 2(c)), a Ca²⁺-ionophore that mobilizes the entire amount of lumenally stored Ca²⁺ by forming Ca²⁺-conducing pores on ER membrane. Hence, a functional SOCE is present in primary cultures of mRCC cells.

3.2. The Molecular Candidates to Mediate SOCE Are Expressed in mRCC Cells. The putative molecular underpinnings of SOCE in mRCC cells were scrutinized by performing a qRT-PCR analysis of their total mRNA content. We focused on Stim1-2, Orai1–3, TRPC1, and TRPC3–7. TRPC2 is a pseudogene in humans and has not been investigated. All the transcripts investigated were readily detectable (Figures 2(d)–2(f)). Single bands of the expected size of cDNA fragments were amplified, as previously shown in [11]. Negative controls were performed by omitting the reverse transcriptase (not shown). The comparison of ΔCt values of the mRNAs obtained by qRT-PCR showed that Stim1 and Stim2 were equally expressed (Figure 2(d)); conversely, Orai3 expression is about fourfold less than Orai1 and Orai2 isoforms (Figure 2(e)), while TRPC3, 4, 6 levels are about threefold less than TRPC1 (Figure 2(f)). Similarly, TRPC5 is about 300 fold less expressed than TRPC1, while TRPC7 is
Figure 2: Store-operated Ca\textsuperscript{2+} entry is present in primary cultures of mRCC cells. The “Ca\textsuperscript{2+} add-back” protocol revealed that emptying the intracellular Ca\textsuperscript{2+} pool with CPA (10 μM; (a)), thapsigargin (2 μM; (b)), or ionomycin (5 μM; (c)) in the absence of extracellular Ca\textsuperscript{2+} (0Ca\textsuperscript{2+}) led to a robust increase in [Ca\textsuperscript{2+}]\textsubscript{i} upon Ca\textsuperscript{2+} restoration to the bath, which is the hallmark of SOCE. Quantitative real-time reverse transcription polymerase chain reaction of total RNA performed by using specific primers revealed that transcripts encoding for Stim1-2 (d), Orai1–3 (e), and TRPC1–7 (f) are expressed in mRCC cells. Bars represent the mean ± SEM of at least 4 different experiments each from different RNA extracts (see Section 2 for details). * and ‡P < 0.05 (one-way ANOVA followed by Newman-Keuls’ Q Test).
nearly absent (Figure 2(f)). In order to confirm transcript expression at protein level, we carried out a number of western blot experiments by utilizing affinity-purified antibodies selectively targeting Stim1, Stim2, Orai1, Orai3, TRPC1, and TRPC3/6/7 [10, 31, 32]. Since TRPC7 transcript is barely expressed, the latter signal is likely to be generated by TRPC3 and TRPC6. As depicted in Figure 3, immunoblots showed a major band of about 33 kDa for Orai1 and Orai3 (Figure 3(b)), whereas Stim1 and Stim2 displayed a doublet of about 77 and 100 kDa (Figure 3(a)), and TRPC1 and TRPC3/6/7 exhibited major bands of about 110 kDa (Figure 3(c)). The band sizes were in agreement with those previously observed by using the same antibodies in circulating EPCs [10, 31, 32]. Therefore, mRCC cells are endowed with all the molecular candidates to mediate the store-dependent Ca$^{2+}$ influx induced by CPA, thapsigargin, and ionomycin.

3.3. Pharmacological Profile of SOCE in mRCCs. SOCE in naïve cells may be inhibited by the pyrazole derivative BTP-2 (20 μM), low concentrations of lanthanides (e.g., 1-10 μM
Figure 4: Store-operated Ca\textsuperscript{2+} entry is not sensitive to BTP-2 and Pyr 6 in mRCC cells. Biphasic Ca\textsuperscript{2+} response evoked by CPA ((a); 10 \( \mu \)M), thapsigargin (2 \( \mu \)M; (c)), or ionomycin (5 \( \mu \)M; (e)) in mRCC cells pretreated with BTP-2 (20 \( \mu \)M) for 20 min. Statistical analysis conducted on 80–90 cells per conditions revealed that BTP-2 did not affect the peak amplitudes of both Ca\textsuperscript{2+} release and Ca\textsuperscript{2+} entry stimulated by CPA (b), thapsigargin (d), or ionomycin (f). Similarly, 5 min preincubation with Pyr6 (10 \( \mu \)M) did not affect CPA-induced intracellular Ca\textsuperscript{2+} discharge and SOCE in mRCC cells (g). (h) Statistical evaluation of the effect exerted by Pyr6 on the peak amplitudes of both Ca\textsuperscript{2+} release and Ca\textsuperscript{2+} entry stimulated by CPA.

Gd\textsuperscript{3+}, and the membrane permeable synthetic drug 2-aminoethyldiphenyl borate (2-APB) [4, 25, 31, 34]. Unexpectedly, BTP-2 (20 \( \mu \)M; 20 min) did not affect either Ca\textsuperscript{2+} release or SOCE occurring in response to CPA (10 \( \mu \)M) in mRCC cells (Figures 4(a) and 4(b)). Similarly, BTP-2 (20 \( \mu \)M; 20 min) did not reduce the amplitude of intracellular Ca\textsuperscript{2+} release and extracellular Ca\textsuperscript{2+} inflow induced by either thapsigargin (2 \( \mu \)M; Figures 4(c) and 4(d)) or ionomycin (5 \( \mu \)M; Figures 4(e) and 4(f)). These results strongly suggest that neither Orai1 nor TRPC1, which are dramatically sensitive to this drug, contribute subunits to the conducting pore of store-dependent channels in mRCC cells [3, 4]. Then, we probed the effects of 10 \( \mu \)M Gd\textsuperscript{3+}, which selectively abrogates Ca\textsuperscript{2+} entry through Orai1 and TRPC1 [3]. Our preliminary experiments demonstrated that preincubating the cells for 30 min with 10 \( \mu \)M Gd\textsuperscript{3+} did not produce any significant reduction in both CPA- and thapsigargin-induced SOCE \( (n = 84 \text{ and } n = 96, \text{ resp.; data not shown}). \) Likewise, Pyr 6 (10 \( \mu \)M, 5 min), a recently synthesized specific Orai blocker did not affect the biphasic Ca\textsuperscript{2+} response to CPA \( (n = 82; \text{Figures 4(g)-4(h)}). \) Therefore, we turn on to 100 \( \mu \)M Gd\textsuperscript{3+}, which exerts an unspecific inhibition on TRPC-gated Ca\textsuperscript{2+} inflow. At this dose, Gd\textsuperscript{3+} suppressed SOCE in response to either CPA (Figures 5(a) and 5(b)) or thapsigargin (Figures 5(c) and 5(d)), without affecting intracellular Ca\textsuperscript{2+} release (Figures 4(a)–4(d)). Then, we ascertained
3.4. Weak InsP₃-Dependent Ca²⁺ Signalling in mRCCs. Then, we sought to assess whether InsP₃ signalling is coupled to SOCE activation in mRCC cells. The thiol-reactive agent thimerosal, which serves as InsP3R agonist in a variety of cell types, has widely been employed to stimulate InsP3-dependent Ca²⁺ release [37]. However, 25 μM thimerosal was ineffective in the large majority of cells tested (Figures 7(a) and 7(c)), while it induced a brief burst of Ca²⁺ spikes only in a modest fraction of cells when its concentration was raised up to 50 μM (Figures 7(b) and 7(c)). In order to assess whether the drug was indeed capable of stimulating InsP₃-dependent Ca²⁺ release, we probed its effects on EPCs isolated from healthy donors, which served as positive control. Twenty-five μM thimerosal evoked repetitive oscillations in intracellular Ca²⁺ concentration ([Ca²⁺]ᵢ) in ~70% of cells (Figures 7(b) and 7(c)), which testify for the efficacy of
Figure 6: 2-Aminoethoxydiphenyl borate and carboxamidotriazole block store-operated Ca\(^{2+}\) entry in mRCC cells. (a) 2-APB (50 \(\mu\)M, 20 min of pre-incubation) selectively inhibited CPA (10 \(\mu\)M)-induced SOCE, but not intracellular Ca\(^{2+}\) mobilization. (b) Statistical analysis of the effect exerted by 2-APB on the peak amplitudes of CPA-induced Ca\(^{2+}\) release and Ca\(^{2+}\) entry. (c) CAI (10 \(\mu\)M, 20 min of preincubation) reduced both phases of the Ca\(^{2+}\) response to CPA (10 \(\mu\)M). (d) Statistical analysis of CAI-dependent inhibition of CPA-elicited Ca\(^{2+}\) release and CPA-elicited Ca\(^{2+}\) entry. * \(P < 0.05\) (Student’s \(t\) test).

the drug batch we employed. This issue was further investigated by challenging mRCC cells with the InsP\(_3\)-synthesizing transmitter, ATP, which binds to purinergic P\(_{2Y}\) receptors to engage PLC-\(\beta\) and activate SOCE [10, 11]. As shown in Figure 7(d) (grey tracing) and Figure 7(e), 100 \(\mu\)M ATP was never able to induce Ca\(^{2+}\) mobilization from ER in mRCC cells, albeit in parallel experiments it induced InsP\(_3\)-dependent Ca\(^{2+}\) discharge and SOCE in most EPCs (black tracing in Figures 7(d) and 7(e)). These results could be explained by the lack of InsP\(_3\)Rs in mRCC cells. However, qRT-PCR analysis unveiled that these cells are present with the transcripts encoding for all the three known isoforms of InsP\(_3\)Rs (i.e., InsP\(_3\)R1-3; Figure 7(f)) [37], the relative pattern of expression being InsP\(_3\)R3 > InsP3R2 > InsP3R1. InsP\(_3\)R expression was further probed by immunoblotting, which revealed a large band, deriving from the sum of 313/260/250 kDa bands, for InsP\(_3\)R1/2/3. These data were confirmed by administrating SDF-\(\alpha\) (10 ng/mL), the most important chemoattractant cytokine involved in cancer dissemination, which induces SOCE upon InsP\(_3\)-dependent mobilization of intraluminally stored Ca\(^{2+}\) [38]. SDF-\(\alpha\) (10 ng/mL) elicited a transient elevation in [Ca\(^{2+}\)]\(_i\) in a minor proportion of mRCC cells (Figures 8(a) and 8(c)). This pattern of Ca\(^{2+}\) signalling is consistent with a modest intracellular Ca\(^{2+}\) release, but not with a sizeable Ca\(^{2+}\) influx [16]. Conversely, SDF-\(\alpha\) (10 ng/mL) elicited a rapid Ca\(^{2+}\) peak which rapidly decayed to a plateau phase of intermediate amplitude, which is the typical hallmark of SOCE [16], in EPCs (Figures 8(a) and 8(b)). Accordingly, the “Ca\(^{2+}\) add-back” protocol revealed that SDF-\(\alpha\)-induced SOCE was
Figure 7: InsP₃-dependent signalling in mRCC cells and endothelial progenitor cells. (a) Thimerosal triggered a brief train of intracellular Ca²⁺ spikes in mRCC cells when applied at 50 μM (black tracing), while it was almost ineffective at 25 μM (grey tracing). (b) Prolonged oscillations in intracellular Ca²⁺ levels stimulated by thimerosal (same solution as that used in (a), recording performed on the same day) in EPCs isolated from mRCC patients. (c) Percentage of mRCC cells and EPCs responding to thimerosal. *p < 0.05 (Student’s t-test). (d) 100 μM ATP stimulated InsP₃-dependent Ca²⁺ mobilization from EPCs (black tracing), but not from mRCC cells (grey tracing). The same solution was used in both experiments and the recordings were conducted on the same day. (e) Fraction of mRCC cells and EPCs responding to ATP. *p < 0.05 (Student’s t-test). (f) InsP₃-R transcripts in primary mRCC cells. *p < 0.05 versus InsP₃-R₁, †p < 0.05 versus InsP₃-R₂. The inset illustrates InsP₃-R expression detected by an InsP₃-R-I/II/III antibody. A major band that corresponds to the sum of the three single 313/260/250 kDa bands for InsP₃-R₁/2/3 was found.
Figure 8: Stromal derived factor 1-α (SDF-1α), vascular endothelial growth factor (VEGF), and foetal bovine serum (FBS) do not trigger store-operated Ca\(^{2+}\) entry in mRCC cells. (a) Fraction of cells responding to SDF-1α in mRCC cells and EPCs. (b) SDF-1α (10 ng/mL), produced a Ca\(^{2+}\) transient (black tracing) in mRCC cells while it elicited a biphasic increase in [Ca\(^{2+}\)]\(_i\), which is the hallmark of SOCE activation, in EPCs. (c) SOCE was triggered by SDF-1α (10 ng/mL) in EPCs, but not in mRCC cells. (d) 2-APB (50 μM), CAI (10 μM), Gd\(^{3+}\) (100 μM) did not inhibit mRCC cell proliferation. Inset, lower, and higher doses of 2-APB and CAI did not impair mRCC cell proliferation. (e) Brief elevation in [Ca\(^{2+}\)]\(_i\) induced by VEGF (100 ng/mL) in mRCC cells. (f) VEGF (10 ng/mL) ignites repetitive Ca\(^{2+}\) oscillations in EPCs. (g) Fraction of cells responding to VEGF in mRCC cells and EPCs. (h) 20% foetal bovine serum (FBS) elicits a biphasic elevation in [Ca\(^{2+}\)]\(_i\) in EPCs, but not mRCC cells.
3.5. Store-Operated Ca\textsuperscript{2+} Entry Does Not Control Proliferation in mRCC Cells. The metastatic RCC cells utilized in the present study have been isolated from malignant lesions and have, thus, already been exposed to the chemotactic clues that drive them to the target organ from the primary tumour. In the search for an alternative target to eradicate disseminated metastases from the patients, we reasoned it was more appropriate to focus on SOCE involvement in mRCC proliferation. Consequently, we probed the effect of 100 \( \mu \)M Gd\textsuperscript{3+}, 50 \( \mu \)M 2-APB, 20 \( \mu \)M CAI, and 10 \( \mu \)M, as negative control, on mRCC proliferation. Figure 8(d) clearly shows that none of these compounds inhibited cell growth after 7 days in culture; therefore, SOCE is not required in the replication process in these cells. Among the signalling pathways utilized by growth factors to promote cell cycle progression, store-dependent Ca\textsuperscript{2+} inflow stands out as a prominent mechanism [4, 13, 14, 25, 39]. In agreement with the lack effect of SOCE inhibition on mRCC cell proliferation, 20% FBS did not generate any detectable increase in \([\text{Ca}^{2+}]_i\) (\(n = 65\), data not shown). Moreover, 10 ng/mL VEGF elicited a modest Ca\textsuperscript{2+} transient in a small fraction of cells (Figures 8(e) and 8(g)), while it induced robust intracellular Ca\textsuperscript{2+} oscillations in EPCs (Figures 8(f) and 8(g)) due to the concerted interaction of InsP\textsubscript{3}-dependent Ca\textsuperscript{2+} release and SOCE [9]. The fraction of responding mRCC cells augmented when VEGF concentration was raised up to 100 ng/mL (Figure 8(g)), but the Ca\textsuperscript{2+} signal still lacked a plateau phase (not shown). Similarly, 20% FBS activated a biphasic Ca\textsuperscript{2+} signal in EPCs (grey tracing in Figure 8(h); \(n = 55\)), but not in mRCC cells (black tracing in Figure 8(h); \(n = 62\)). Finally, EGF did not ignite any detectable Ca\textsuperscript{2+} activity when administrated at 10 ng/mL (\(n = 74\), respectively; data not shown), which has been shown to trigger a sustained Ca\textsuperscript{2+} inflow in other cell types [40]. Overall, these data argue against the requirement for SOCE to drive proliferation in mRCC cells. We finally sought to ascertain whether extracellular Ca\textsuperscript{2+} inflow is involved in mRCC cell growth. The cells were plated in complete culture medium and, after three days, external Ca\textsuperscript{2+} was chelated by the addition of 5 mM EGTA. As depicted in Figure 9, this treatment dramatically prevented mRCC from replicating. Therefore, albeit SOCE does not control this process, mRCC proliferation requires extracellular Ca\textsuperscript{2+} entry.

4. Discussion

Store-operated Ca\textsuperscript{2+} entry is among the most widespread mechanisms of Ca\textsuperscript{2+} entry in cancer cells, thereby contributing to regulate a growing number of processes involved in malignant transformation, including cell proliferation, differentiation, and metastatization [4, 12–14, 25, 27, 39, 41–45]. It is, therefore, not surprising that SOCE has been put forward as a promising target to develop alternative therapies of metastatic tumours. We have recently found that the pharmacological blockage of SOCE suppresses cell proliferation in circulating EPCs isolated from naïve mRCC patients [10]. Therefore, we hypothesized that SOCE inhibition could provide an alternative strategy to promote eradicate metastases in subjects resistant to multit kinase inhibitors [3]. To reinforce this concept, we undertook the present investigation to assess whether SOCE drives proliferation in primary cultures of mRCC cells isolated from these patients.

The “Ca\textsuperscript{2+} add-back” manoeuvre revealed that a functional SOCE is present in mRCC cells and is activated upon pharmacological emptying of ER Ca\textsuperscript{2+} content [10, 11]. Growing body of evidence indicates that SOCE in cancer cells is mediated by Stim1, which relays the information relative to the drop in ER Ca\textsuperscript{2+} levels to cell periphery, where it binds to and activates Orai1, a highly Ca\textsuperscript{2+}-selective pore-forming unit [10, 13, 14, 17, 20, 21, 42, 44]. Alternatively, SOCE might
require the involvement of a less Ca\textsuperscript{2+}-selective membrane pathway, such as that provided by TRPC channels. In more detail, all TRPC channels may participate in SOCE either through direct binding to Stim1 (i.e., TRPC1, TRPC4, and TRPC5) or upon multimerization with Stim1-gated isoforms (i.e., TRPC3 and TRPC6). In particular, TRPC1 and TRPC6 have been involved in SOCE-mediated proliferation and cytokinesis in a number of cancer types [18, 22–24, 42, 45]. The scenario becomes even more complicated when considering that Orai3 is activated by Stim1 upon intracellular store depletion in some oestrogen receptor-positive breast cancer lines [21], and that Orai may assemble into a supramolecular ternary complex with Stim1 and TRPC1 [46]. In the present investigation, we utilized qRT-PCR analysis to demonstrate that mRCC cells possess all the molecular candidates to mediate SOCE, that is, Stim1-2, Orai-3, TRPC1, TRPC4, TRPC5, and TRPC6. However, Orai3 is far less expressed than Orai and Orai2, whereas TRPC1 is more abundant than TRPC3, TRPC4, TRPC5, and TRPC6. Immunoblotting has confirmed that mRCC cells present with Stim1-2, Orai3, TRPC1, and TRPC6 proteins. It is worth noting that mRCC cells present with TRPC3 and TRPC5, which are absent in normal renal epithelium and in RCC cell lines [47]. This feature supports the notion that established long term cell cultures may not truly represent the biological complexity of a real metastatic phenotype. These expression data hint at the participation of Stim1-2, Orai1-2, and TRPC1 to SOCE activation in mRCC cells. This hypothesis is corroborated by two pieces of evidence. First, endogenous TRPC6 mediates SOCE and sustains proliferation in Hep G2 and Huh-7 human hepatoma cells [18, 19], in human and rat glioma cell lines [50] and in human gastric cancer cells [51]. Second, TRPC6 expression is enhanced in a variety of primary neoplasms as compared to normal paratumour tissues [50–52], including RCC [53]. Third, TRPC6 is sensitive to CAI in Hep G2 and Huh-7 human hepatoma cells [19], albeit this drug may also prevent intracellular Ca\textsuperscript{2+} mobilization in mRCC cells. The effect exerted by CAI both on Ca\textsuperscript{2+} release and on store-dependent Ca\textsuperscript{2+} influx is a consequence of the inhibition of mitochondrial Ca\textsuperscript{2+} uptake, as shown in HEK293 cells [54]. Despite the pharmacological and molecular evidence of TRPC6 involvement in SOCE, we cannot rule out the contribution of Orai proteins or other TRPC channels, as part of TRPC6-containing complexes [15, 46]. For instance, TRPC4 would be indispensable to confer store sensitivity to TRPC6 [15], which is otherwise gated by diacylglycerol [48]. Moreover, over-expressed Orai proteins may assemble with endogenous TRPC channels in a Stim1-dependent manner to form a SOCE pathway which is insensitive to low micromolar Gd\textsuperscript{3+} [55]. Therefore, further studies are required to untangle the molecular architecture of SOCE in mRCC cells.

Store-operated Ca\textsuperscript{2+} entry is normally activated upon InsP\textsubscript{3}-dependent depletion of the intracellular Ca\textsuperscript{2+} stores [4]. A peculiar feature of SOCE in mRCC cells was its weak coupling to InsP\textsubscript{3} signalling. These cells are endowed with all the three known InsP\textsubscript{3} R isoforms, their pattern of mRNA expression being InsP\textsubscript{3} R3 > InsP\textsubscript{3} R2 > InsP\textsubscript{3} R1, which is similar to that found in GMB [56] and breast carcinoma [57]. Our results revealed that InsP\textsubscript{3}-dependent Ca\textsuperscript{2+} release is loosely coupled to SOCE. Thimerosal oxidizes thiols to form a thiomercuration complex, thereby sensitizing InsP\textsubscript{3} to basal InsP\textsubscript{3} levels [37]. In our hands, up to 50 \mu M thimerosal was almost ineffective at mobilizing intracellular Ca\textsuperscript{2+} in mRCC cells, while it induced repetitive Ca\textsuperscript{2+} oscillations in EPCs. These observations suggest that the resting concentration of InsP\textsubscript{3}, which is attributable to constitutive PLC activity, is low. When we sought to physiologically increase intracellular InsP\textsubscript{3} levels, we found that both ATP and SDF-\textalpha fail to reproducibly mobilize intracellularly stored Ca\textsuperscript{2+} in mRCC cells; the same results were obtained when PLC\textgamma was recruited to the plasma membrane by stimulating TKRs with either VEGF, EGF, or FBS (as discussed below). ATP, FBS, and EGF were actually unable to induce Ca\textsuperscript{2+} release from ER in the totality of the cells probed. It appears that mRCC cells are reluctant to generate InsP\textsubscript{3}-dependent Ca\textsuperscript{2+} signals and to activate SOCE. Indeed, both VEGF and SDF-\textalpha trigger transient elevations in [Ca\textsuperscript{2+}], which lack the plateau phase caused by persistent SOCE activation. InsP\textsubscript{3} metabolism may be dramatically deranged in cancer cells. It has recently been demonstrated that the activity of the InsP\textsubscript{3} producing enzymes, phosphatidylinositol (PI) 4-kinase, PI
4-phosphate-5-kinase, and PLC, is enhanced in neoplastic tissues, while the catabolic enzymes, PIP, 5-phosphatase and PIP 4-phosphatase, are downregulated; this is likely to lead to an amplification of InsP$_3$-related pathways [58]. It could be hypothesized that the opposite occurs in mRCC cells and is responsible for the weak InsP$_3$ signalling we observed. An alternative, but not mutually exclusive, explanation includes the downregulation of the membrane receptors we have tried to exploit or their lower sensitivity to ligand binding in these cells (see below).

The present study aimed to assess whether the pharmacological blockade of SOCE leads to the inhibition of primary cultures established from metastatic lesions of patients resistant to the most recent anti-angiogenic therapies. Nevertheless, the inhibition of SOCE with Gd$^{3+}$, 2-APB, and CAI did not impair mRCC cell proliferation. The lack of effect of CAI deserves a careful evaluation. This is a synthetic small molecule compound that inhibits a Ca$^{2+}$-mediated cellular responses by exerting a nonspecific block on Ca$^{2+}$ entry and release pathways in both malignant cells and tumour vessels [4, 25]. CAI is currently under investigation as an orally administered tumourstatic and antiangiogenic agent in clinical phase I–III trials of several solid cancers [25], including RCC [4, 25]. As reviewed in [25], this agent was supposed to possess antitumour activity against mRCC based on the observation, coming from phase I trials, of cases of disease stabilization and minor responses. Unfortunately, a randomized discontinuation trial of CAI in mRCC patients was not successful, the compound being inactive and not well tolerated. The present investigation permits understanding the failure of CAI administration at cellular level as this drug did not affect mRCC cell proliferation in our hands. To the best of our knowledge, this is the first report that CAI does not hamper cell cycle progression in tumour cells [41]. SOCE is the ubiquitous mechanism whereby Ca$^{2+}$ inflow drives cell cycle progression and DNA synthesis in both normal and cancer cells. The "habitation" of mRCC cells to reduced SOCE is, therefore, the most remarkable finding of the present study. This feature renders RCC cells, which are freshly isolated from metastatic effusions and maintained in culture for less than 3-4 passages, different from the commercially available immortal tumour cell lines, which heavily rely on SOCE to proliferate [18–23, 44, 45, 50, 51]. Curiously, a recent investigation conducted on primary cultures established from GBM disclosed that Stim1/Orai account for ~20% of cell growth [17], thereby confirming that freshly isolated tumour cells may become independent on SOCE to proliferate. It is reasonable to speculate that the sharp fall in SOCE requirement to proliferate may be considered a novel mechanism of tumour resistance to pharmacological treatments that could be identified only in patients-derived cells. Consistent with these results, recent work has convincingly demonstrated that external Ca$^{2+}$ entry through either store-operated or arachidonate-activated channels does not support proliferation in three different tumoural cell lines, such as HEK293, HeLa, and Huh-7 cells [59], and in EPCs harvested from patients affected by primary myelofibrosis [32]. In order to explain our result, it is mandatory to recall that mRCC cells have been cultured from metastatic lesions of patients who progressed after being exposed to at least two VEGF/VEGF(Rs) inhibitors and one mTOR inhibitor. These drugs are administrated to disrupt the vascular network which sustains the exceeding tumour growth and paves the way for the dissemination of malignant deposits throughout the organism [3]. The physiological stimulus that activates SOCE to control cell proliferation is the InsP$_3$-dependent drop in ER Ca$^{2+}$ levels which is consequent to TKR stimulation by extracellular growth factors [9, 23, 45, 50]. The prolonged exposure to multikinase inhibitors, which target the intracellular pathways downstream VEGF, PDGF, and EGF receptors, might "silence" the ability of metastatic cells to respond to endogenous growth factors [60], albeit the treatment was designed to impair endothelial signalling. As a consequence, either the reduced sensitivity to ligand binding or the inhibition of TKR activation would fail to mobilize a sufficient amount of Ca$^{2+}$ from the InsP$_3$-sensitive store. Future work is required to assess whether store-dependent Ca$^{2+}$ inflow governs cell proliferation in mRCC cells isolated from naïve patients, that is, who are yet to undergo any pharmacological treatment. Accordingly, EPCs isolated from these subjects cannot expand in the presence of SOCE inhibitors [10]. Two pieces of observations should, however, be borne in mind in regard to the role of Ca$^{2+}$ signalling in mRCC cell growth. First, although the influx of Ca$^{2+}$ is not important to drive proliferation in HEK293, HeLa, and Huh-7 cells, the same investigation unveiled that the genetic suppression of Orai1 and Orai3 impeded mitosis progression in these settings [59]. Therefore, these protein channels fulfill cellular functions other than conducting signalling ions across the plasma membrane that are relevant to cell proliferation [59, 61]. Second, whereas the pharmacological blockade of SOCE did not cause any significant decrease in mRCC cell number after 5 days of incubation, buffering external Ca$^{2+}$ with EGTA blocked cell proliferation. As a consequence, this process is controlled by a different Ca$^{2+}$ entry pathway(s) and these cells do not habituate to extracellular Ca$^{2+}$ deprivation [26]. A variety of additional Ca$^{2+}$-permeable may control cell growth in nonexcitable cells, such as receptor-operated channels (ROCs), second messengers-operated channels (SMOCs), voltage operated-channels (VOCs), and ionotropic receptors [16]. One or more of these conductances are likely to regulate mRCC cell proliferation and should be identified in future experiments.

5. Conclusions

This investigation demonstrated for the first time that a functional SOCE is present in primary cultures of mRCC cells isolated from patients treated with multikinase inhibitors. The pharmacological charaterization and the molecular screening of Stim, Orai, and TRPC transcripts are consistent with the involvement of TRPC6. A role for Stim, Orai, and other TRPC isoforms cannot, however, be ruled out: in fact, these proteins tend to assemble in supramolecular heteromeric complexes which display different pharmacological, ion selectivity, and gating properties as those observed when
ectopically expressed as single units. SOCE is loosely coupled to InsP$_3$-dependent signalling and cannot be activated by ATP, SDF-1α, VEGF, FBS, and EGF. Consistent with this observation, the pharmacological blockade of SOCE does not affect mRCC proliferation. These results suggest that SOCs are unlikely to provide a suitable molecular target to design alternative treatments for subjects resistant to multikinase inhibitors. Caution is, therefore, warranted when SOCE inhibition is put forward as a novel option for cancer therapy exclusively on the basis of results obtained from immortal tumour cell lines.

**Disclosure**

Daniela Montagna and Francesco Moccia share senior authorship of the paper.

**Conflict of Interests**

The authors declare that there is no conflict of interests regarding the publication of this paper.

**Authors’ Contribution**

Silvia Dragoni and Ilaria Turin equally contributed to this work.

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