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Oncogenic KRAS suppresses store-operated Ca^{2+} entry and I_{CRAC} through ERK pathway-dependent remodelling of STIM expression in colorectal cancer cell lines

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ABSTRACT

The KRAS GTPase plays a fundamental role in transducing signals from plasma membrane growth factor receptors to downstream signalling pathways controlling cell proliferation, survival and migration. Activating *KRAS* mutations are found in 20% of all cancers and in up to 40% of colorectal cancers, where they contribute to dysregulation of cell processes underlying oncogenic transformation. Multiple KRAS-regulated cell functions are also influenced by changes in intracellular Ca^{2+} levels that are concurrently modified by receptor signalling pathways. Suppression of intracellular Ca^{2+} release mechanisms can confer a survival advantage in cancer cells, and changes in Ca^{2+} entry across the plasma membrane modulate cell migration and proliferation. However, inconsistent remodelling of Ca^{2+} influx and its signalling role has been reported in studies of transformed cells. To isolate the interaction between altered Ca^{2+} handling and mutated KRAS in colorectal cancer, we have previously employed isogenic cell line pairs, differing by the presence of an oncogenic *KRAS* allele (encoding $KRAS^{G13D}$), and have shown that reduced Ca^{2+} release from the ER and mitochondrial Ca^{2+} uptake contributes to the survival advantage conferred by oncogenic KRAS. Here we show in the same cell lines, that Store-Operated Ca^{2+} Entry (SOCE) and its underlying current, I_{CRAC} are under the influence of $KRAS^{G13D}$. Specifically, deletion of the oncogenic *KRAS* allele resulted in enhanced STIM1 expression and greater Ca^{2+} influx. Consistent with the role of KRAS in the activation of the ERK pathway, MEK inhibition in cells with $KRAS^{G13D}$ resulted in increased STIM1 expression. Further, ectopic expression of STIM1 in HCT 116 cells (which express $KRAS^{G13D}$) rescued SOCE, demonstrating a fundamental role of STIM1 in suppression of Ca^{2+} entry downstream of $KRAS^{G13D}$. These results add to the understanding of how ERK controls cancer cell physiology and highlight STIM1 as an important biomarker in cancerogenesis.

1. Introduction

The small GTPase RAS is a molecular switch that couples receptor tyrosine kinase (RTK) activation with downstream signalling pathways regulating cell proliferation, survival and migration. Somatic mutations at several levels in these pathways, for example in RTKs (EGFR), RAS itself, BRAF and PI3 K, are highly prevalent in cancer and are responsible for the constitutive activation of affected pathways observed [1]. Notably, underlining its importance in oncogenic transformation, *RAS* is mutated in 20–30% of all cancers and up to 40% of colorectal cancer (CRC), which is the second and third most prevalent cancer in women and men, respectively, affecting over one million patients per year globally [2]. Of the three RAS isoforms, *KRAS* is most frequently mutated in cancer, and as the only essential isoform it has the greatest impact on cell biology [1]. The importance of growth factor signalling pathways in oncogenic transformation has placed them at the centre of cancer drug development efforts. To date however, while drugs targeting RTKs, BRAF, MEK and PI3 K are at various stage of development or in use in the clinic, no effective drugs targeting KRAS are available [3].

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Constitutive activation of KRAS in cancer is brought about through missense point mutations in the codons encoding Gly12, Gly13 or Gln61 [1]. Consequently, the activities of pathways downstream of KRAS are increased. KRAS signals via four main pathways: the RAF/MEK/ERK pathway, the PI3 K/AKT pathway, the RAL pathway and the PLCε/PKC/Ca^{2+} pathway. These pathways do not act in isolation however but signal in concert. Notably, Ca^{2+} signalling pathways and the KRAS-RAF-MEK-ERK pathway interact at many levels [4,5]. For example, inhibition of BRAF increases the expression of Ca^{2+} ATPase isoform 4b (PMCA4) [6] and facilitates endoplasmic reticulum (ER)-mitochondrial Ca^{2+} transfer [7]. Upon phosphorylation, KRAS interferes with Bcl_{XL} interaction with and sensitisation of $InsP_3Rs$, influencing Ca^{2+} signalling [8]. Notably, Ca^{2+} signals also feedback to regulate RAS activity [9].

Like KRAS, Ca^{2+} signals have pleiotropic effects controlling cellular life and death decisions [4]. As such, dysregulated Ca^{2+} signalling pathways contribute to the altered activity of cell processes underlying cancer [4,5,10]. In particular, Ca^{2+} signals sustain the cell cycle at G_1/S and G_2/M transitions [4,11,12] and induce apoptosis via mitochondrial Ca^{2+} overload [4,10]. Ca^{2+} signals also regulate the cell migration and invasion associated with tumour metastasis [13–15].

Increases in cytosolic Ca^{2+} are generated through release from intracellular stores and/or influx across the plasmalemma. While Ca^{2+} signalling pathways centred on the endoplasmic reticulum (ER) have primarily been invoked in the altered cell physiology of cancer cells, a prominent role for Ca^{2+} influx across the plasmalemma is now also emerging [16]. Indeed, the Ca^{2+} influx pathway engaged following depletion of the ER Ca^{2+} store (Store-Operated Ca^{2+} Entry; SOCE) contributes to the regulation of cell proliferation, migration and apoptosis [16] – cell processes modified in cancer cells [4,5,17]. Further supporting a role for SOCE in these processes, altered SOCE and expression and activity of its underlying mediators STIM and ORAI are observed in cancer, including in colorectal cancer [18,19] and melanoma [15,20]. Indeed, STIM1 and ORAI1, have been found to control G_1/S transition in cervical cancer SiHa cells [11]. Moreover, inhibition of STIM1 and ORAI1 via siRNA impairs migration of breast [21,22] and cervical cancer cells [23], and overexpression of STIM1 favours invasion [18].

Despite these advances in identifying altered intracellular Ca^{2+} regulation as a hallmark of cancer, the interaction between Ca^{2+} homeostasis/signalling and cellular transformation induced by a particular oncogenic mutation remains poorly explored. While analysis of tumour-derived and normal cells of the same tissue origin or neighbouring to the tumour has provided important information [15,19], these controls may not adequately reflect the cell type/genetic status of the cell type of origin. Further inconsistencies are observed on analysis of transformed cells from tumours at different stages and origins as well as whether the original tumour or its metastasis are analysed [24,25]. An approach successfully used to circumvent these issues in cellular diversity has been to determine the consequences of genetic deletion of specific mutated oncogenic alleles from a transformed cell [26,27]. Comparison of isogenic cell line pairs thus generated provides specific information regarding the interaction between the mutated allele and the phenotype studied. Given the frequency of *KRAS* mutations in colorectal cancer, we have used this approach to probe functional interactions between this GTPase and Ca^{2+} handling. We previously demonstrated that through suppression of inositol 1,4,5-trisphosphate receptor ($InsP_3R$) expression, cells harbouring a mutated *KRAS* allele exhibited a reduction in both Ca^{2+} signalling induced by an $InsP_3$-generating agonist and of mitochondrial Ca^{2+} uptake, which together served to protect them from death-inducing stimuli [28]. Given the growing body of evidence supporting a role of Ca^{2+} influx in cell proliferation and cell migration in cancer, including in CRC, we here examined whether Ca^{2+} entry mechanisms were also a target of activated KRAS. As previously, we analysed CRC cell line pairs that were isogenic albeit for a single copy of oncogenic *KRAS* (encoding $KRAS^{G13D}$), which was deleted by homologous recombination [26]. Use of these cell line pairs thus allows the selective analysis of the influence of the oncogenic KRAS on cell phenotype without the confounding effects observed in studies in which KRAS is expressed at supraphysiological levels, such as induction of cell senescence [27]. Using biochemical, fluorometric and electrophysiological approaches, we demonstrate that $KRAS^{G13D}$ expression in the isogenic CRC cell model was associated with reduced SOCE and I_{CRAC} as well as remodelled expression of STIM proteins. $KRAS^{G13D}$ expression was also associated with a reduced sensitivity to cell death induced by activation of SOCE. The lower Ca^{2+} entry in $KRAS^{G13D}$ expressing cells was augmented to the levels in the KRAS-deficient cells by STIM1 overexpression, indicating that STIM1 expression is a direct downstream target of $KRAS^{G13D}$ in CRCs. Targeting KRAS/Ca^{2+} signalling interactions pathway may thus provide a strategy to intervene in the development of CRC.

2. Methods

2.1. Materials

Salts for physiological recordings were of the highest grade and purchased from Sigma-Aldrich, Fisher Scientific or BDH. Na-methanesulfonate, Adenosine 5′-triphosphate magnesium salt and Cs-methanesulfonate and salts for internal solutions were from Sigma (St. Louis, MO, USA). Cs-BAPTA and Ca^{2+}-sensitive fluorescent indicators were from Invitrogen (Eugene, OR, USA). Sources for other reagents used are indicated where described.

2.2. Cell culture

HCT 116 and DLD-1 cells (both $KRAS^{G13D/WT}$) and their respective isogenic derivatives HKH-2 and DKO-4 (both $KRAS^{-/WT}$) were a kind gift of S. Shirasawa (Fukuoka University, Japan) and have been previously described [26]. Cells were cultured in DMEM (Life Technologies, Carlsbad, CA, USA), containing 10% heat-inactivated foetal bovine serum (FBS) (Invitrogen), 1% penicillin/streptomycin solution (5 units penicillin, 55 µg streptomycin) (Sigma, Dorset, UK). Cells were maintained at 37 °C with 5% CO_2 in saturated humidity and passaged upon reaching 80–90% confluency.

2.3. Imaging of cytosolic Ca^{2+}

Imaging and analysis of cytosolic Ca^{2+} levels was performed as previously described [28]. Briefly, cells were seeded onto poly-L-lysine-coated coverslips at equivalent densities and imaged after 48 h. Imaging was carried out using Ca^{2+} containing and Ca^{2+} free HEPES buffered solutions as previously described [29] and included in the supplementary information. Prior to each experiment, coverslips were mounted into stainless steel imaging chambers and loaded with fura-2 AM diluted in Ca^{2+} containing imaging buffer (Life Technologies; 2 µM for 30 min), followed by de-esterification in Ca^{2+}-free buffer for 30 min during which thapsigargin (Tg, 2 µM; SIGMA) was included for the final 15 min. Coverslips were imaged on the stage of a Nikon Eclipse TE200 inverted epifluorescence microscope equipped with a Nikon PlanFluor 20x/0.75 NA multi immersion objective (Nikon, Kingston Upon Thames, Surrey, UK). Excitation light at 340 and 380 nm was selected using a motorised filter wheel (Sutter Industries, Novato, CA, USA) at a frequency of 1 image pair every 3 s with an exposure of 200 ms and emitted light was selected using a 400 nm dichroic mirror and filtered through a 460 nm long pass filter. Images were captured using a Hamamatsu ORCA ER Charge-Coupled Device (CCD) camera. Three coverslips per cell type were imaged per day on 3 separate days.