Interaction between Lamin A/C and Src mechanosensors in the nucleus of osteosarcoma cells: implications for diagnosis, prognosis and therapy

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Abstract

Mechanotransduction is the process by which cells can convert extracellular mechanical stimuli into biochemical changes within a cell. In cancer cells, this mechanism can be completely deregulated, thereby participating in cancer onset, progression and metastatization. In our previous work, we demonstrated that SaOS-2 osteosarcoma cancer cells, commonly recognized as low aggressive osteoblast-like cells, exhibit high nuclear Lamin A/C expression compared to more-aggressive osteosarcoma cell lines. Interestingly, the nuclear content of Src, a tyrosine-kinase involved in key cellular processes such as cell proliferation, migration and mechanosensing, has the same behaviour of Lamin A/C and decreases as osteosarcoma cell aggressiveness increases. Based on literature evidence about the importance of lamins phosphorylation, we tested whether Src interacts with Lamin A/C in the nucleus of SaOS-2 cells. Here, we demonstrated a tight relationship between Lamin A/C and Src in SaOS-2 cell nuclei, assessed by advanced imaging-based microscopy techniques. With confocal laser scanning and STED microscopy, a statistically significant co-distribution between the two proteins was observed, especially in the nuclear rim rather than in the nuclear matrix. To deepen the Src-Lamin A/C colocalization at the nanoscale level, we used the time-domain fluorescence lifetime imaging microscopy (FLIM), a sensitive molecular imaging method to detect protein-protein interactions, combined with Forster's resonance energy transfer (FRET) detection (FLIM-FRET technique), demonstrating a close relationship between Src and Lamin A/C in the nuclear compartment of osteosarcoma cells. These findings suggest to consider osteosarcoma as a mechanopathology, thereby opening to new therapeutic approaches for patients. Understanding how the physical microenvironment influences cancer cell signalling will lead to the improvement of strategies to target the deregulated mechano-sensitivity of osteosarcoma cells.

Results

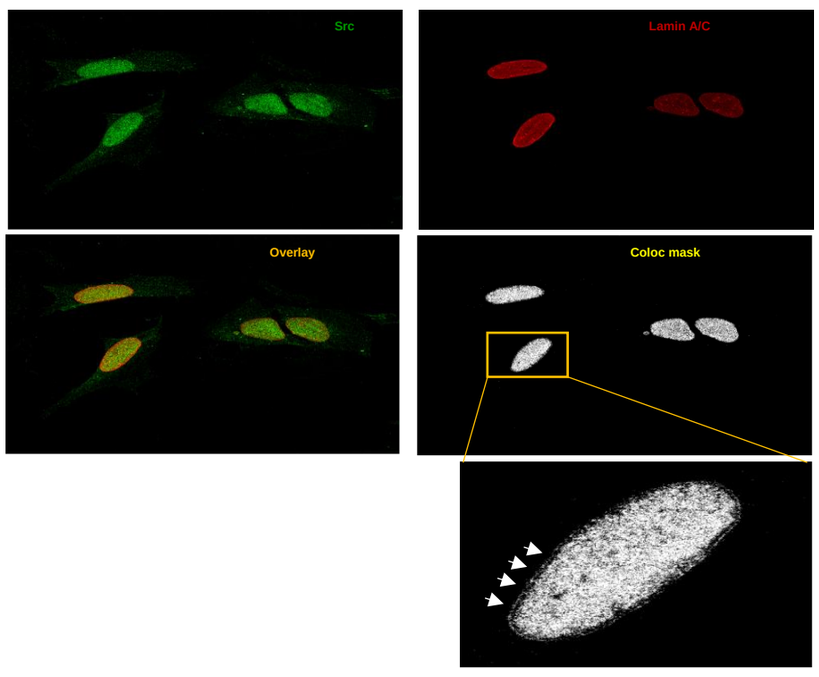

Figure 1. Confocal microscopy imaging of Src (in green) and lamin A/C (in red) in SaOS-2 cells. Src immunofluorescence showed a punctate and diffuse distribution in the nuclear and cytoplasmic compartments, with a higher concentration in the nuclei. White pixels showed the overlay of Src and Lamin A/C fluorescence in doubled-stained cells. Colocalization masks of double-stained cells (white pixels) showed the Src-lamin A/C co-distribution both at the nuclear envelope (arrows) and in the nucleoplasm.

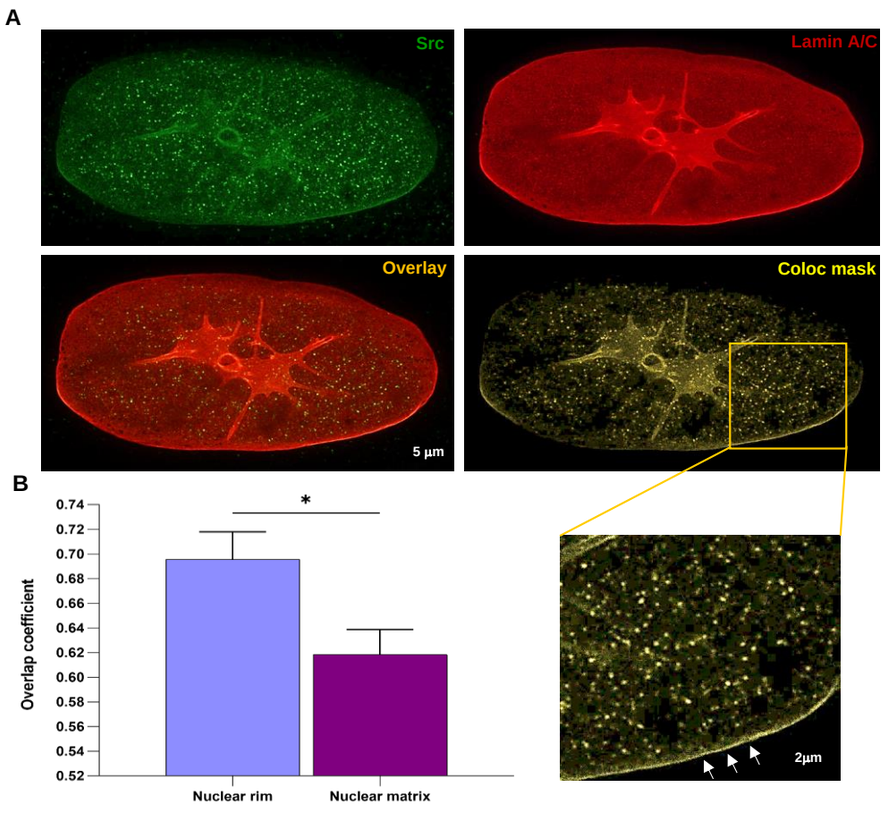

Figure 2 - A) STED nanoscopy of Src and lamin A/C in SaOS-2 nuclei. Alterations in the structural organization of the lamin A/C (red) meshworks have been seen in several nuclei of SaOS-2 cells population. Colocalization masks (yellow) showed the co-distribution of Src and lamin A/C at the nuclear rim in and in the nuclear matrix. Bars: 5 µm and 2 µm. **B)** Mean values of the Overlap Coefficient quantified in STED images of double-stained ssaOS-2 cell nuclei (*p < 0.05).

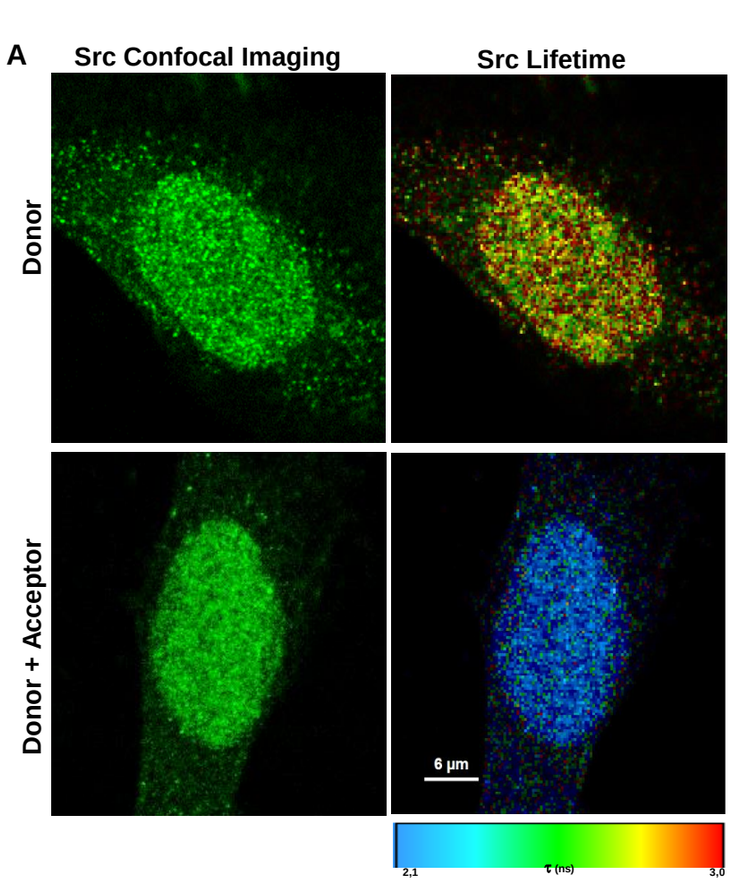

Figure 3. FLIM and FLIM-FRET microscopy of Src and lamin A/C in SaOS-2 cells.
A) Fluorescence lifetime imaging of Src-AF488 donor in absence (τD, left panel) or in presence (τDA, right panel) of lamin A/C-AF594 acceptor. Different τ values were visualized by color code lifetime scale bar (from 2.0 to 3.0 ns of range). **B)** Selected ROI in lifetime image **B')** Src-AF488 mean lifetime values in absence of the acceptor (τD), showed a significant increase in specific region of SaOS-2 nuclei, in the graph is evidence how the diffuse region has a lifetime significantly increased in comparison to the dotted sites. **C)** Src-AF488 donor lifetime in the presence of the acceptor molecule (τDA) was significantly decreased in all the analyzed cells. **D)** Quantified FRET efficiency values (mean ± sem) of the Src-AF488 and lamin A/C-AF 594 pair obtained in all selected ROIs

Discussion

Mechanotransduction refers to the ability of cells to translate extracellular mechanical cues into intracellular biochemical responses. In cancer cells, this process may become profoundly dysregulated, contributing to tumor initiation, progression, and metastasis. We know that Src, a non-receptor tyrosine-kinase, is strongly expressed into the nuclei of SaOS-2 cells and that these cells present nuclear dysmorphism. Src is involved in mechanosensing and its cytoplasmic functions are very well known and characterized, while poor is known about Src role into nuclear compartment. We previously demonstrated that Src follows the same behaviour as Lamin A/C in osteosarcoma cells with different aggressiveness and that laminopathic nuclei show an aberrant interaction between Src and LaminA/C in comparison to healthy fibroblast. We tested whether this association occurs also into SaOS-2 cell line. Here, we provide the first evidence of a molecular interaction between Src and Lamin A/C at the level of the nuclear lamina in osteoblast-like cells. The FLIM-FRET technique has become an essential tool in cell biology and biomedical imaging for studying: protein-protein interactions, conformational changes, protein complex dynamics and many others. FRET describes the transfer of energy from an excited donor fluorophore to a neighbouring acceptor molecule when they are in close proximity (<10 nm). The application of the FRET-FLIM technique, which benefits from fluorescence lifetime being independent of donor fluorophore concentration, gives us stronger results than STED or classic confocal microscopy. Further studies are required to clarify the role of the Src–Lamin A/C interaction and to determine whether it may have a prognostic value in osteosarcoma.

Materials and Methods

Cell culture

Human osteosarcoma cell lines SaOS-2 were purchased from Banca Biologica and Cell Factory (IRCCS Azienda Ospedaliera Universitaria San Martino-IST, Genova, Italy). Cells were maintained in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich, Saint Louis, MO) supplemented with 10% Fetal Bovine Serum (FBS), 5 U/ml penicillin, and 5 µg/ml streptomycin (Hyclone) at 37°C with 5% CO2.

Immunofluorescence

5x103 cells were seeded into plastic-chambered glass microscope slides (BD Falcon) and fixed with 4% paraformaldehyde in PBS for 10 min followed by PBS/Triton 0,1% for 5 min. The following antibodies were used according to manufacturer instructions: primary rabbit anti c-Src and mouse anti-lamin A/C antibodies were used diluted in 1% PBS/BSA overnight, at 4°C. Secondary goat immunoglobulins conjugated with Alexa Fluor 488 and 594 dyes have been used for confocal microscopy acquisitions. Slides were mounted with PBS/glycerol 1:1. In parallel for STED experiments, primary anti-Src and lamin A/C were revealed using goat anti-rabbit and goat anti-mouse conjugated to STAR Orange and STAR Red dyes, respectively and slides were mounted using antifade mounting medium.

Confocal Microscopy imaging

Confocal microscopy was performed on a Leica TCS-SP8X laser-scanning confocal microscope equipped with tunable white light laser (WLL) source, 405 nm diode laser, 3 Internal Spectral Detector Channels (PMT, including 2 dedicated to single molecule detection (SMD), and 2 Internal Spectral Detector Channels (HyD) GaAsP. Sequential confocal images were acquired using HC PL APO 40x/oil immersion (1.30 NA) and 63x/oil (1.40 NA) objectives. The mean fluorescence intensities of Src and lamin A/C were manually measured in each nucleus, then calculated using MetaMorph software. A number >80 nuclei from seven digital images (40x magnification) was randomly selected and analyzed for each cell sample, and the experiments were repeated twice. Colocalization analysis was performed using Huygens Colocalization Analyzer to obtain the overlap coefficient value between the fluorophore pair.

Super-resolution STED microscopy

STED imaging was performed on a STED microscope platform equipped with an upright Zeiss Axioimager Z.2 microscope, an oil-immersion objective lens was used (100x, 1.46 NA), and a pulsed STED laser with a depletion wavelength of 775 nm working at a repetition rate of 40 MHz, a pulse duration of 1 ns, using STEDYCON SmartControl software version 9. SaOS-2 cells were immunostained using Src and Lamin A/C antibodies and revealed with specific goat secondary antibodies conjugated to the dyes STAR Red and STAR Orange respectively. STAR RED was imaged with a pulsed source at a wavelength of 640 nm (detection range between 650-700 nm, gated detection between 1 and 7ns), whereas STAR Orange was excited with a pulsed source at 561 nm (detection range between 575-625 nm, gated detection between 1 and 7ns). The pinhole set to 1.1 Airy Units at 650nm. Colocalization analysis was performed using Huygens Colocalization Analyzer (SVI) to evaluate the overlap coefficient (n=10 of nuclei for each sample were analyzed).

FLIM and FRET-FLIM

The mean fluorescence lifetime (t) of the fluorophore conjugated to Src antibody (AF488) was detected by time-domain FLIM on a pixel-by-pixel basis. FLIM measurements were calculated in specific selected ROIs as follows: at the nuclear rim where lamin A/C is polymerized and in matrix regions where Src showed a dotted distribution. The FLIM data were visualized in the lifetime image wherein each pixel was represented with a color code corresponding to a specific t value (in the indicated scale bar). Best fitting results were obtained for estimating the donor fluorescence lifetime in the absence of the acceptor (tD, unquenched donor lifetime), by applying a mono-exponential decay model using the n-exponential reconvolution fitting approach, and the quality of the fit was judged by the residual distribution and by the goodness of $\chi 2$ value (close to 1). Lifetime t and relative amplitude A of the individual exponential components were obtained from the SymPhoTime64 software (PicoQuant, Berlin, Germany). By applying a bi-exponential decay, the donor fluorescence lifetime in the presence of the acceptor, using the donor lifetimes only, the values of tAV AMP (amplitude-weighted average lifetime) were obtained using SymPhoTime64 software. The FRET efficiency (E) was calculated for each ROI using the equation: E = 1- (tAV AMP/tD), where tAV AMP is the amplitude-weighted average lifetime of the donor in the presence of the acceptor and tD is the donor in the absence of the acceptor.