Use of focused ion beam/scanning electron microscopy (FIB/SEM) to study senescent features of pluripotent stem cells

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Understanding structural and compositional details down to the nanoscale is paramount to any scientific purpose, ranging from materials science to biomedical research. Focused ion beam/scanning electron microscopy (FIB/SEM) is a recently developed technology used by research laboratories, nanotechnology centers, and companies worldwide. Dualbeam instrument combines one electron beam (SEM column) and one ion beam (FIB column), oriented at 52°, and focused on the same point of the sample, thus enabling the operator to selectively ablate in a nanometer scale a previously marked region of the sample by using a focused ion current from a gallium source. The milling process can be interrupted every few nanometers to take high-resolution images of cross sections by the SEM column. Despite the great potential and flexibility of this innovative approach, applications are so far mainly limited to particle analysis and material characterization, being only occasionally utilized to study biological samples.
Induced pluripotent stem cells (iPSCs) represent a most promising tool in biomedical research, in that they mimic embryonic stem cell features, while being obtained from adult tissues. Indeed, they display the ability to differentiate in virtually any cell type, while being capable to mitotically divide, self-renewing themselves. Owing to these pluripotency and self-renewal properties, human iPSCs constitute an unlimited source of patient-specific cell types that could be used for disease modeling, as well as for tailored cell-based therapies, particularly concerning regenerative medicine and drug screening [1]. Human iPSCs are promptly obtained from skin biopsies, by reprogramming fibroblasts, through a recently established *in vitro* procedure, involving non-integrating episomal technology. Though extensively characterized from a molecular/genetic point of view, ultrastructural studies dealing with iPSCs peculiar features are still few, encouraging further research addressing this issue. Even scarcer information is presently available on the possible ultrastructural changes occurring in iPSCs, as a function of culturing time. In fact, despite general assumption that iPSCs can be maintained and propagated indefinitely in culture, alterations involving mitochondrial biogenesis and function have recently been found in long-term cultured iPSCs [2].
To investigate the issue of senescence processes occurring in iPSCs, we comparatively analysed ultrastructural features of iPSCs maintained in culture for 1, 6 and 12 month, focusing on mitochondrial morphology and intracellular distribution.
Human iPSCs derived by healthy male adult fibroblasts were cultured on Matrigel (BD Biosciences) in mTeSR1 (Stemcell Technologies) and passaged for varying time. They were transferred to Chamber Slide™ (Lab-Tek®), fixed in 0.2% glutaraldehyde and 2% paraformaldehyde in 0.1M cacodylate buffer and embedded in epoxy resin, prior to being analysed by a FIB/SEM Helios Nanolab (FEI Company, Eindhoven, The Netherlands). Resin-embedded iPSCs were mounted on stubs by using a self-adhesive carbon disk and gold sputtered by an Emithech K550. Regions of interest were cross-sectioned by the focused gallium ion beam operated at 30 kV and 6.5 nA. Pictures of each cross-section were acquired at a working distance of 2 mm using backscattered electrons (BSE) and a through-the-lens (TDL) detector in immersion mode with an operating voltage of 2 kV and an applied current of 0.17 nA (Fig. 1).
FIB/SEM micrographs from 1-month-cultured iPSCs show few mitochondria displaying spherical, immature-like shape, poorly developed cristae and mostly perinuclear localization. At difference, 6- and 12-month-cultured iPSCs display structurally mature mitochondria, widely distributed in the cytoplasm (Fig.2). These organelles show an elongated tubular morphology, numerous distinct cristae, reminiscent of a differentiated phenotype. Moreover, in mid-to-long-term cultured iPSCs, double-membrane limited vacuoles containing mitochondria and other cytoplasmic material, referred to as, autophagosomes are readily recognized (Fig. 2). These structures representing an index of ongoing senescence processes further support the previously hypothesized concept that long-term maintenance of iPSCs in culture may alter mitochondrial status [2]. In view of the crucial role played by mitochondria in the maintenance of pluripotency, differentiation, and reprogramming of iPSCs we suggest that these important properties may be progressively lost during culturing time, with relevant impact on their potential use for cell therapy. Indeed, culturing time-dependent features of iPSCs suggest their use as a faithful model of cell senescence and possibly of age-dependent human diseases.

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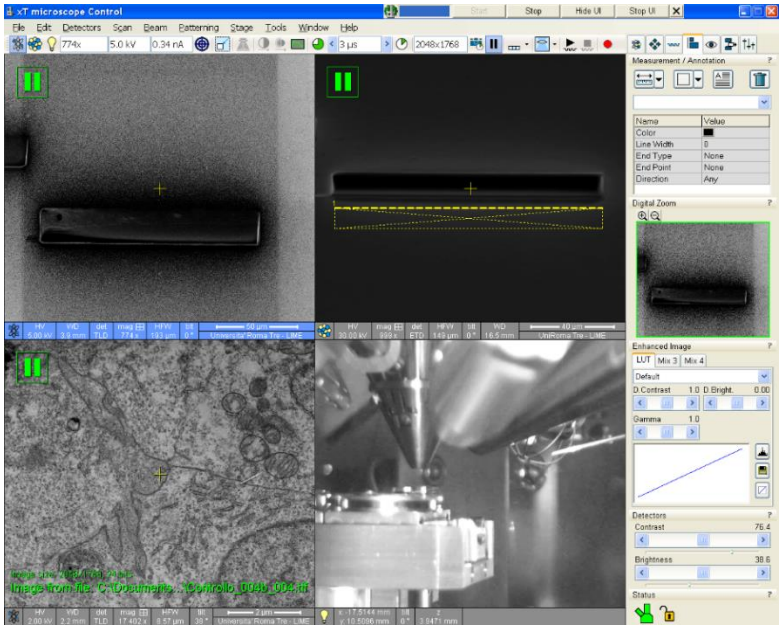

Figure 1. User interface showing SEM image with secondary electrons (top left quad); FIB image (top right quad); SEM image with backscattered electrons (bottom left quad); infrared CCD camera image (bottom right quad).

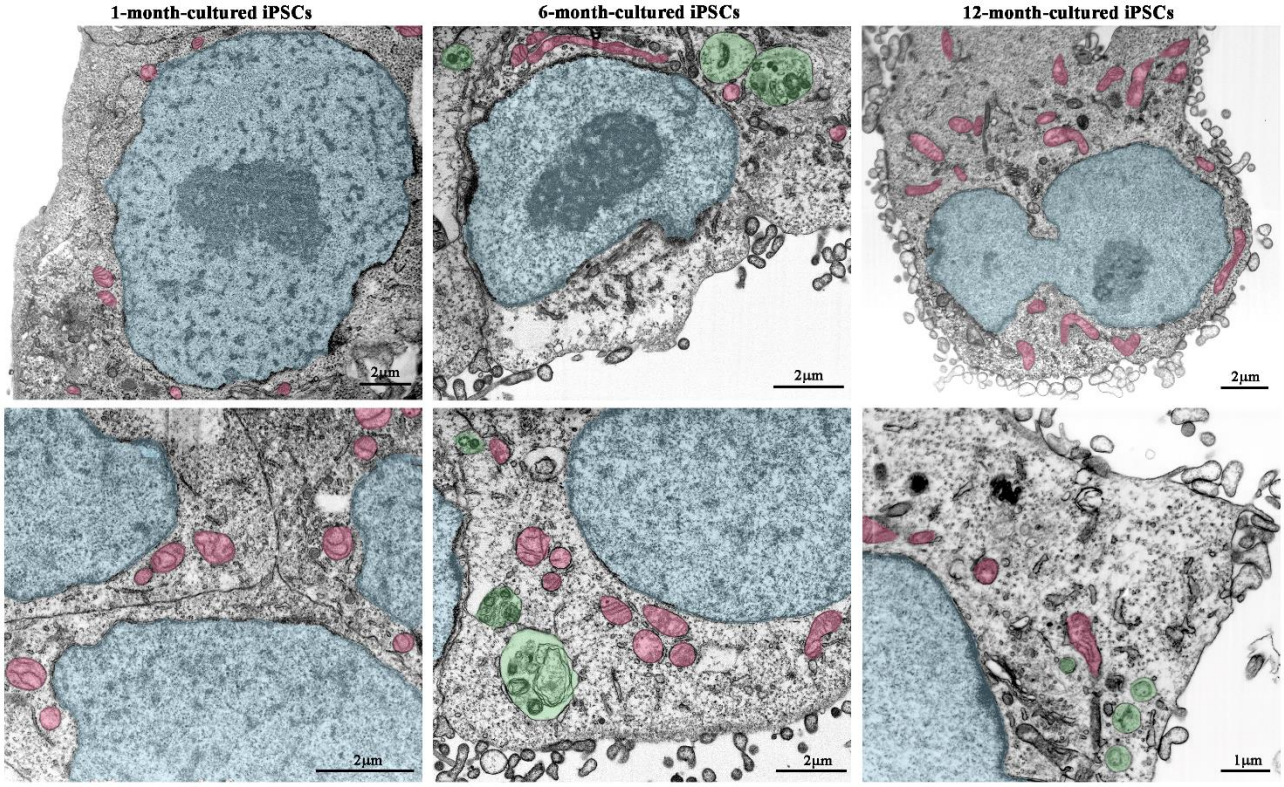

Figure 2. FIB/SEM micrographs from 1-, 6-, 12-month-cultured iPSCs. Light blue, nuclei; pink, mitochondria; green, autophagosomes.