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Lipid rafts integrity is essential for prolactin-induced mitogenesis in mouse embryonic stem cells

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ABSTRACT **Embryonic stem cells, ESCs, retain the capacity to self-renew, yet, the protein machinery essential in maintaining this undifferentiated status remains largely undefined. Signalling interactions are initiated and enhanced at the plasma membrane lipid rafts, within constraints and regulations applied by the actin and tubulin cytoskeleton systems. First, we undertook a comprehensive approach using two-dimensional gel electrophoresis and mass spectrometry analysis combined with Western blotting and immunofluorescence analyses at the single cell level to compile the proteome profile of detergent-free preparations of lipid rafts of E14 mouse embryonic stem cells. In comparison with the proteomic profiles of other membrane fractions, recovery of actin and tubulin network proteins, including folding chaperones, was impressively high. At equally high frequency, we detected annexins, pleiotropic proteins that may bind membrane lipids and actin filaments to regulate important membrane processes, and we validated their expression in lipid rafts. Next, we tested whether lipid raft integrity is required for completion of mitogenic signalling pathways. Disruption of the rafts with the cholesterol sequestering methyl-β-cyclodextrin (MCD) greatly downregulated the mitotic index of ESCs, in a dose- and time of exposure-dependent manner. Moreover, MCD greatly reduced the mitogenic actions of prolactin, a hormone known to stimulate proliferation in a great variety of stem and progenitor cells. Taken together, our data postulate that lipid rafts in ESCs act in close association with the actin and tubulin cytoskeletons to support signal compartmentalization, especially for signalling pathways pertinent to symmetric divisions for self-renewal.**

KEYWORDS: *lipid rafts, annexin, prolactin, stem cells*

Introduction

Embryonic stem cells (ESCs) from the inner cell mass of embryos at the blastocyst stage are capable of unlimited, symmetrical, self-renewing mitoses when established as cell lines in culture (Burdon *et al.,* 2002). However, when re-introduced into a blastocyst, ESCs retain their ability to differentiate into all three embryonic germ layers and generate an embryo (Martin 1980; Brook and Gardner 1997; Thomson and Marshall 1998; Burdon *et al.* 2002). Therefore, as ESCs may provide a renewable cell source for a diversity of conditions, such as traumas, diabetes, myocardial infarction, or neurodegeneration, considerable effort has been made to understand the mechanisms that maintain pluripotentiality or guide ESCs along specific cell lineages. We now know that activation of the Leukaemia Inhibitory Factor (LIF)/Janus kinase 2 (JAK2)/ Signal Transducer and Activator of Transcription Protein 3 (STAT3)

pathway is required to maintain the undifferentiated state of ESCs, as well as certain levels of activation of Protein kinase C, TGFβ/ SMAD, Wnt/β-catenin, and FGF/MEK/ERK pathways, with low levels of ERK activation essential for telomere length maintenance and genomic stability (Chen *et al.* 2015; Huang *et al.* 2015; Ma *et al.* 2016). Despite these extensive studies, we still know little about the subcellular structures involved in the facilitation of signalling complex formation and intracellular propagation of the signal.

Accumulated experimental evidence has highlighted the importance of coalescing lipid rafts as signalling platforms, formed within constrains applied by the underlying cortical filamentous actin (F-actin) cytoskeleton and its associated proteins (Gupta *et al.* 2006; Morone *et al.* 2006; Chichili and Rodgers 2007; Goswami

et al. 2008; Simons and Gerl 2010; Asimaki *et al.* 2011; Asimaki and Mangoura 2011; Karouzaki *et al.* 2019). Lipid rafts represent pre-ordained, liquid-ordered, cholesterol-rich microdomains of the plasma membrane bilayer, with glycosphingolipids and glycosyl phosphatidylinositol−anchored proteins in their outer leaflet, integral transmembrane, palmitoylated proteins (mostly receptors and transmembrane kinases, like JAKs) (Ren *et al.* 2013; Lorent *et al.* 2017), and high concentrations of major lipidated signalling molecules, such as the lipid raft prototype resident H-Ras, Src-family kinases, G proteins, and adaptor molecules in their inner leaflet and cytoplasmic face (e.g., (Asimaki *et al.* 2011; Karouzaki *et al.* 2019)). These specialized concentrations contribute, through lipid-lipid and lipid-protein interactions (Raghupathy *et al.,* 2015), to the generation and dynamic behavior of lipid raft domains (reviewed in (Harayama and Riezman 2018; Kusumi *et al.* 2020)).

More specifically, in quiescent cells, lipid rafts exist as meso-scale domains of 2-20 nm diameter, which float amidst liquid-disordered plasma membrane domains. Upon binding of clustering agents (ligands, antibodies, or toxins, e.g., (Puri *et al.* 2005; Dinic *et al.* 2015)) to their transmembrane receptors, the clustered receptors induce lipid rafts to coalesce and form larger, stabilized membrane domains (up to 300 nm). The liganded receptors might be clustered for longer durations, yet their lipid-anchored signalling effectors move freely and undergo rapid exchanges with those in the lipid-disordered domains. Therefore, the raft domains now function as platforms that facilitate formation of signalling complexes and biomolecular condensates upon membrane receptor stimulation (Simons and Sampaio 2011; Kusumi *et al.* 2012).

Indeed, the significance of biomolecular condensates (i.e. concentrations of macromolecules not surrounded by a membrane) in the biological outcomes of membrane receptor signalling has now become evident (Asimaki and Mangoura 2011; Mangoura 2016; Banani *et al.* 2017; Shin and Brangwynne 2017; Karouzaki *et al.* 2019; Jaqaman and Ditlev 2021; Schuster *et al.* 2021). Such condensates, formed at the plasma membrane upon receptor agonist binding, regulate the activity of signalling proteins, by regulating the time that such molecules spend in the membrane (Huang *et al.,* 2019). Furthermore, these condensates also control actin assembly and remodelling (Case *et al.,* 2019).

While actin cortical structures are remodelled by biomolecular condensates, they also control the lateral movement of lipid rafts (Simons and Gerl, 2010). Specifically, constraints are imposed by F-actin and crosslinking proteins that act as fences, and by interacting proteins, namely cytosolic domains of transmembrane proteins or cytosolic proteins tethered to the membrane through lipid modifications, which act as pickets (Kusumi and Suzuki 2005; Kusumi *et al.* 2012). This extensive network of "fences" and "pickets" forms restraining "corrals" for the movement of lipid rafts through the entirety of the plasma membranes. As F-actin binding proteins are instantly modified by receptor-mediated activation of signalling kinases, the fate of signalling complexes (e.g., (Chen *et al.* 2009; Rollason *et al.* 2009)) and, overall, the spatiotemporal activity of signalling pathways is controlled.

Alpha and beta tubulins, resident proteins in raft microdomains (Ballestrem *et al.* 2000; Li *et al.* 2004), through physical interactions with lipid raft residents flotillin 1, caveolin, and G-proteins are also important functional partners of lipid rafts (Li *et al.* 2004; Dremina *et al.* 2005; Allen *et al.* 2009). Microtubules (MTs), the polymerized dimers of α- and β-tubulin, are highly dynamic in the cell periphery,

and the rapid interchange between elongation and catastrophe is modulated by a number of molecules, mostly microtubule associated proteins (MAPs), within or in close association with lipid rafts. These roles of microtubules and rafts as reciprocal scaffolds have become a focus for the lipid raft role in signal-induced, clathrin-independent endocytosis (Chen and Williams, 2013), and in cell motility and neuritic process outgrowth (Xu *et al.,* 2011). Thorough identification of the molecular protein component of rafts would greatly facilitate these efforts.

Lipid rafts have previously been implicated in embryonic and adult stem cell renewal and expansion (Androutsellis-Theotokis *et al.* 2010; Lee *et al.* 2010) and appear to have particular properties. For example, human ESCs are insensitive to cholera toxin that targets the monosialotetrahexosylganglioside-containing lipid rafts and G_s-proteins, but sensitive to pertussis toxin that targets G_i-proteins (Nakamura *et al.,* 2009). These studies have highlighted the importance of lipid rafts in membrane receptor regulation of ESC self-renewal. However, the protein composition of lipid rafts remains elusive in ESCs (Nagano *et al.* 2005; Wang and Gao 2005; Intoh *et al.* 2009). Therefore, we have sought to analyse the proteome of lipid rafts in two murine ES cell lines - E14 and W4 - as well as to test the functional importance of lipid raft integrity for the mitogenic signalling of prolactin (PRL), an established mitogen for many stem and progenitor cells, including precursor cells in the adult mouse hippocampus (Walker *et al.,* 2012), astroblasts and neuroblastoma cells (Cheng *et al.* 2000; Mangoura *et al.* 2000), as well as many cancer stem cell types (e.g., (Rouet *et al.,* 2010).

Results and Discussion

The proteome profile of lipid rafts from stem cells reveals enrichment in tubulins, actins, and annexin

E14T or W4 ES cells grew with a cell cycle of 18-24 h for the first 3-days post plating, and then mitosis rates fell, most likely due to silencing of bivalent chromatin domains that silence developmental gene, while keeping cells poised for activation upon initiation of differentiation (Bernstein *et al.,* 2006); thus, cultures were usually passaged after 96 h. Both mouse ESC lines (mESC) were routinely monitored by morphology and by semiQ-PCR of stemness markers Nanog and Oct-4 (Fig. 1) to verify their undifferentiated state (McLaughlin *et al.,* 2006), as these transcription factors control pathways that govern pluripotency, self-renewal, genome surveillance and cell fate determination (Loh *et al.,* 2006). Under these conditions, ESCs could be induced to form embryoid bodies (EBs, Fig. 1) and, after treatment of EBs with retinoic acid and replating, to differentiate to neurons (Fig. 1, ESC-N), using established protocols (Bibel *et al.* 2004; McLaughlin *et al.* 2006). As shown in Fig. 1, both Nanog and Oct-4 expression was downregulated in EBs and practically nullified in differentiated neurons. In order to have a nearly synchronized cell population and thus maximum cell protein content homogeneity, we analyzed the proteome of lipid rafts at 60 h post-plating.

Using an optimized detergent-free method that preserves the native lipid and protein composition of the lipid rafts, and allows considerable resolution of lipid rafts, from caveolin-rich domains, and intracellular ER or Golgi membranes (Chambers *et al.* 2003; Asimaki *et al.* 2011; Mangoura 2016; Karouzaki *et al.* 2019), we analyzed enrichments of lipid raft proteins in E14T and W4 ESCs (Fig. 2). Protein content is very low compared with other fractions
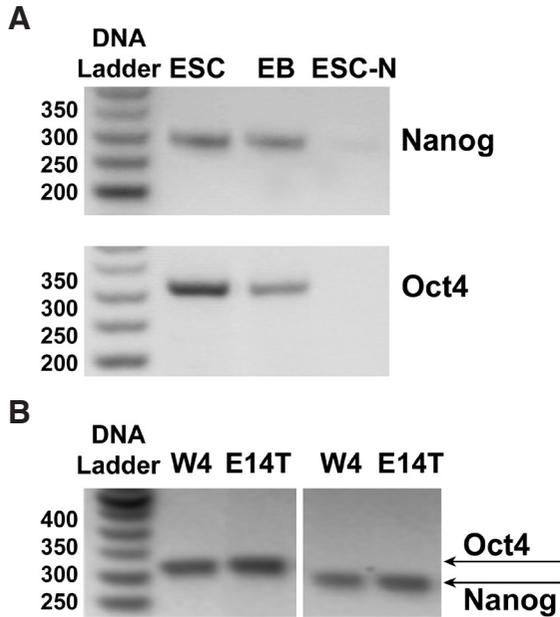
A

B

Fig. 1. Expression of stem cell markers in mouse stem or differentiated cells. (A) mRNA expression levels of Nanog (285 bp) or Oct4 (313 bp), determined by semi-quantitative PCR as described in Methods, is high in E14 ES cells (lanes ESCs); expression of both stemness markers was significantly decreased when ESCs were withdrawn from LIF and induced to form embryoid bodies (lanes EBs), and became undetectable when EBs were neuronally induced with retinoic acid (lanes ESC-N). **(B)** Identical results were obtained with the mESC line W4.

(e.g., Fig. 3 and (Karouzaki *et al.,* 2019)) and therefore we identified and analyzed all visible spots, i.e. 81 spots from 2-DE gels at pH 3-10, 66 of which were positively identified and 28 found to be unique in the lipid raft fractions, as compared with the rest of the membrane fractions (pooled fractions 1-5, Table 1). The ratio of structural proteins was impressively high, as previous studies have also noted (Nagano *et al.,* 2005). Indeed, recovery of actin and tubulin network proteins was truly significant (Table 1). Moreover, the presence of almost all actin isoforms and of T-complex subunits (P80318, P80313, P80317), which are well known molecular chaperons for folding of actin and tubulin, shows that we have isolated both the outer and inner leaflet of lipid rafts, as well as the intimately associated underlying cortical cytoskeleton. This task is almost impossible when detergents are used and these components get separated (Li *et al.* 2004; Morris *et al.* 2004; Persaud-Sawin *et al.* 2009).

Actins and tubulins

The actin cytoskeleton is indispensable for all primal cell processes, i.e. mitosis, cell motility and migration, and regulation of signalling complexes, as is also attested by the fact that mRNAs for β and γ actin are the most abundant species during oogenesis, even increasing between the 8-cell and the blastocyst stage. Hence, mutations of actin genes are lethal in early embryonic stages (Taylor and Piko 1990; Schoenenberger *et al.* 2011). Out of the six actin genes and corresponding proteins (Perrin and Ervasti, 2010), five were present in the lipid rafts of mESCs (P60710, P63268, P68033, P62737, P63260). Four are considered muscle specific, out of which only Acta 1, a skeletal muscle actin, was not detected, while

the cytoplasmic, ubiquitously expressed β- and γ1-actin (Actb and Actg1) isoforms were present. The abundance of actin species may signify a strong compartmentalization of the rafts in the plasma membrane of mESCs.

Equally crucial for such pivotal functions and general cell and organism growth right from the zygote stage are the tubulins, which serve such functions courtesy of their unique ability rapidly to form highly dynamic noncovalent polymers, the microtubules, i.e. polymerized structures of α- and β-tubulin dimers ((Brouhard and Rice, 2018), and refs therein). Both α- and β-tubulin exist as multiple isotypes, encoded by nine different genes each that have several differences in their amino acid sequences (https://www.genenames.org/); these α- and β-tubulins can form dimers (Ludueña, 2008) in a 1:1 stoichiometry (Montecinos-Franjola *et al.,* 2019) and then, in a GTP-dependent manner, polymerize to form protofilaments that eventually assemble into the functional microtubule, i.e. the hollow, 13 protofilament-tube (Mitchison and Kirschner 1984; Chaaban and Brouhard 2017). The way in which different tubulin isotypes assemble to form specialized MT structures, given that the different post-translational modifications (PMTs) imprint on them an additional level of complexity, is now referred to as the "tubulin code", and major research efforts are dedicated to resolving this for each function served, i.e. from mitotic spindle formation to neuronal axon transport (Ferreira *et al.* 2018; Janke and Magiera 2020). For these reasons, tubulins are notoriously difficult to detect proteomically, requiring in-solution digestion and detection with HPLC-MS/MS (Li *et al.,* 2004), yet our protocols successfully detected at least most β isotypes. Alpha tubulins were

TABLE I

MOUSE EMBRYONIC STEM CELLS PROTEINS DETECTED WITH MS IN DETERGENT-FREE LIPID RAFT PREPARATIONS

Uniprot ID	Protein Full Name	Gene Name
P62737 (ACTA_MOUSE)	Actin, aortic smooth cell	Acta2
P60710 (ACTB_MOUSE)	Actin, cytoplasmic 1	Actb
P68033 (ACTC_MOUSE)	Actin, α cardiac muscle	Actc1
P63260 (ACTG_MOUSE)	Actin, cytoplasmic 2	Actg1
P63268 (ACTH_MOUSE)	Actin, γ enteric smooth	Actg2
P07356 (ANXA2_MOUSE)	Annexin A2	Anxa2
P14824 (ANXA6_MOUSE)	Annexin A6	Anxa6
Q07076 (ANXA7_MOUSE)	Annexin A7	Anxa7
Q03265 (ATPA_MOUSE)	ATP synthase subunit α, mitochondrial	Atp5f1a
P56480 (ATPB_MOUSE)	ATP synthase subunit β, mitochondrial	Atp5f1b
P10126 (EF1A1_MOUSE)	Elongation Factor1 α1	Eef1a1
P08113 (ENPL_MOUSE)	Endoplasmin	Hsp90b1
P62874 (GBB1_MOUSE)	Guanine nucleotide-binding protein, b1 subunit	Gnb1
P63017 (HSP7C_MOUSE)	Heat shock cognate 71kDa protein	Hspa8
P17742 (PPIA_MOUSE)	Peptidyl-propyl-cys-trans isomer	Ppia
P09103 (PDIA1_MOUSE)	Protein Disulfide-isomerase	P4hb
P52480 (KPYM_MOUSE)	Pyruvate kinase isozymes M1/M2	Pkm
Q7TMM9 (TBB2A_MOUSE)	Tubulin β-2A chain	Tubb2a
Q9CWF2 (TBB2B_MOUSE)	Tubulin β-2B chain	Tubb2b
P68372 (TBB4B_MOUSE)	Tubulin β-2C chain	Tubulin β-2C chain
Q9ERD7 (TBB3_MOUSE)	Tubulin β-3 chain	Tubb3
Q9D6F9 (TBB4A_MOUSE)	Tubulin β-4A chain	Tubb4a
Q3TFB6 (TBB5_MOUSE)	Tubulin β-5 chain	Tubb5
Q922F4 (TBB6_MOUSE)	Tubulin β-6 chain	Tubb6
P80318 (TCPG_MOUSE)	T-complex protein 1 subunit γ	Cct3
P80313 (TCPH_MOUSE)	T-complex protein 1 subunit η	Cct7
P80317 (TCPZ_MOUSE)	T-complex protein 1 subunit ζ	Cct6a

Proteins uniquely identified in detergent-free lipid rafts, as compared with other membrane fractions of mouse Embryonic Stem Cells.

not detected proteomically, as those have different trypsinization profiles, while their PMTs, such as the unique acetylation on Lys40 (Portran *et al.,* 2017), may have interfered. Nevertheless, immunocytochemistry with an antibody that recognizes all α-tubulins (Fig. 4A) decorated MTs (in a pattern identical to the one seen with an antibody that recognizes all beta tubulins, data not shown) in the cell periphery (Fig. 4A, yellow arrow), the cytosol, and the spindle (Fig. 4A single and double arrows, respectively).

The subtypes of β-tubulin identified were β2A, β2B, β2C, β3, β4, β5, β6, (Fig. 2 and Table 1, Q7TMM9, Q9CWF2, P68372, Q9D6F9, Q3TFB6, Q922F4). Beta 2 Tubulins are highly expressed in neurons (Leandro-Garcia *et al.* 2010; Breuss *et al.* 2017; Hausrat *et al.* 2021), and we detected all three forms: β2A, β2B, and β2C. Beta 2 Tubulins were previously thought to overlap functionally; recent genotype-phenotype correlations, however, describe discrete human tubulinopathies (Sferra *et al.* 2018; Schmidt *et al.* 2021; Schroter *et al.* 2021). These, along with different phenotypes after mouse isotype-gene editing (Bittermann *et al.,* 2019), support novel functional differences of the isotypes (Schmidt *et al.,* 2021). The β3 isotype forms very dynamic MTs and was originally thought to be exclusively neuronal, but its high expression is now considered to be both a cancer stem cell marker and a marker of drug (taxane) resistance of solid tumor cells (e.g., (Sekino *et al.,* 2020)). Hence, β3 may possibly participate in the typical epithelial-to-mesenchymal transition of cancer cells and in cancer metastasis processes (Sobierajska *et al.,* 2016). Moreover, β3 is highly expressed in avian neural crest cells (Chacon and Rogers, 2019), the transient, stem-like cell population which gives rise to a range of cell types, from peripheral neurons to melanocytes. In ESCs, β3 tubulin was
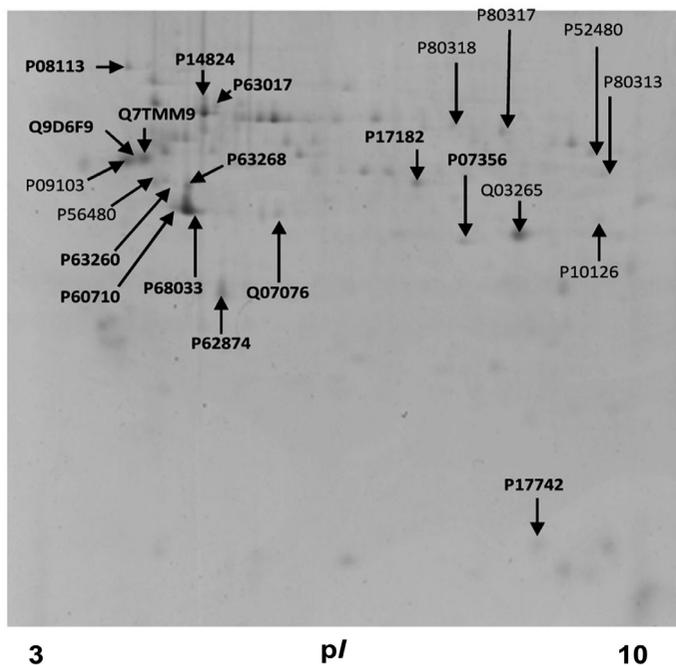

Fig. 2. **Representative two-dimension gel image of proteins from E14 stem cell lipid rafts.** Proteins from detergent-free preparations of lipid rafts (pooled fractions 1-5), were separated with SDS-PAGE on 12% polyacrylamide gel and stained with Coomassie Brilliant Blue. Arrows indicate protein spots that were processed and the accession number and position of identified proteins.
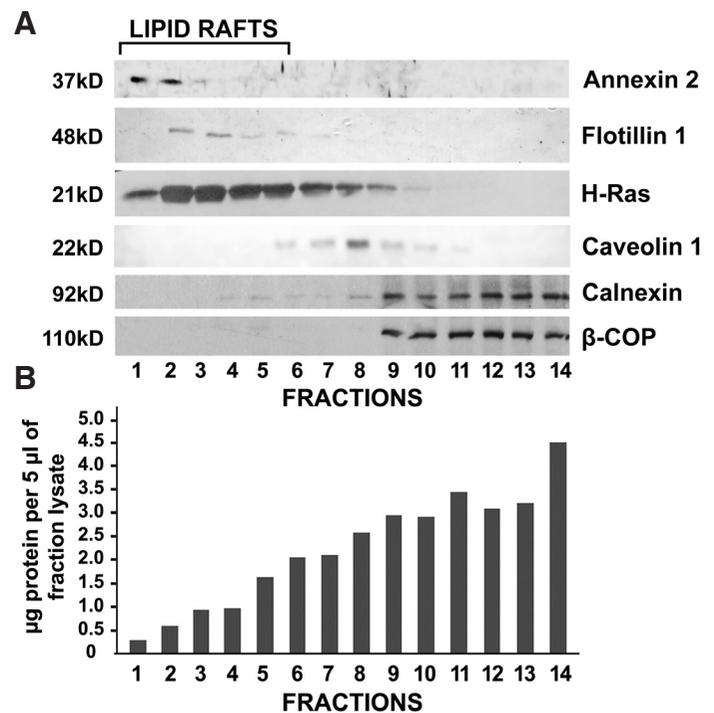

Fig. 3. **Annexin 2 is highly enriched in ESC lipid rafts. (A)** Subcellular fractions were prepared without detergent, as described in Materials and Methods, and 50µl of each of the 14 OptiPrep gradient fractions in Laemmli buffer was analyzed by SDS-PAGE and Western blotting; frames contain scans of typical Western blots with indicated antibodies. Annexin 2 was detected abundant in lipid rafts (>90% of its total expression in cell membranes), also marked as such by the detection of the raft resident proteins flotillin 1 and H-Ras. Conversely, caveolin 1 was detected in the immediately adjacent membrane fractions, and calnexin, an ER protein marker, and β-COP, a Golgi marker, were distributed almost exclusively in denser fractions of the gradient. The M*r* of each protein is indicated on the left-hand side. **(B)** Characteristic protein content distribution of detergent-free cell membrane fractions, showing significantly less protein in the lipid rafts.

detected immunocytochemically in unattached microtubules in the cell periphery (Fig. 4B, yellow arrow) but not in microtubule organizing centres, and decorated the mitotic microtubules (Fig. 4B, double arrows). Indeed, the rapid dynamics of β3 would be very useful in the mitotic spindle, which assembles and de-assembles in very short times frames (< 40 min (Araujo *et al.,* 2016)) in these rapidly dividing cells. Therefore, expression of β3 in ES cells may reflect its role in serving ESC stemness and high proliferation rate. Finally, it is noteworthy that β1-tubulin, an isotype with very low affinity for the actin cytoskeleton, was not detected.

Thus, lack of detergent in our preparations allowed the preservation of protein-protein physical interactions of tubulins with actin filaments (Tsvetkov *et al.,* 2007), an event we also detected with immunocytochemistry, confocal imaging, and colocalization quantification in single focal planes using Volocity, as described in Materials and Methods (Fig. 4C). Indeed, the average colocalization coefficient of 0.288 + 0.05 of actin and alpha tubulin at the cell periphery (Fig. 4C, yellow arrow and graph) is comparable to those with detergent (Peta *et al.,* 2020). The actin cytoskeleton is involved in defining the trajectory of microtubules through a family

of microtubules plus-end–tracking proteins (+TIP proteins) such as CLASPs and members of the spectraplakin protein family, such as ACF7 (Kodama *et al.* 2003; Tsvetkov *et al.* 2007). Other proteins may also contribute to the presence of tubulins in the rafts, such as caveolin-1, a scaffold protein that resides in the inner leaflet of some raft-like domains (Fig. 3). Overexpression of caveolin leads to increased polymerization of microtubules by inhibition of stathmin (Kawabe *et al.,* 2006), and its low expression in ESCs (Fig. 3A) may also indicate that the microtubule network that underlies ESC lipid rafts is highly dynamic.

Annexins

We also identified several annexins - A6 (P14824), A7 (Q07076), and A2 (P07356) - as ESC lipid raft resident proteins (Fig. 2 and Table 1). Annexins are soluble, hydrophilic proteins that may self-associate and bind to negatively charged phospholipids, in a Ca^{2+}-dependent manner, and to F-actin (Raynal and Pollard, 1994). Annexin A2, in

particular, most likely provides a structural link between lipid rafts and the cortical cytoskeleton, since treatment with cholesterol-sequestering agents results in its specific release together with the cortical cytoskeletal proteins α-actinin, ezrin, and actin (Harder *et al.,* 1997). Annexin A6 has been shown to promote a cysteine protease-dependent remodelling of the membrane cytoskeleton and may facilitate receptor-mediated budding of clathrin-coated vesicles at the plasma membrane during endocytosis (Smythe *et al.,* 1994). Annexin A7 is involved in mediating the energy-Ca^{2+}-dependent exocytotic membrane fusion, and is pivotal for early embryo development as its deletion in mice leads to embryonic lethality (Gerke and Moss, 2002). We did not detect A5, an annexin that has been suggested as a differentiation marker in rat ST14A progenitor cells (Hoffrogge *et al.,* 2007).

Importantly, Western blotting analysis of annexin 2 in all fractions validated the strong enrichment of the protein in the lipid rafts (Fig. 3). As expected, the lipid raft marker flotillin 1 (Macdonald and Pike, 2005) was concentrated in the same fractions, while H-Ras, another well-established lipid raft marker, was also detected there; caveolin 1, as expected, was faintly detected in fraction 5 of the lipid rafts and more prominent in fractions 6-8. In contrast, calnexin and β-COP, two proteins associated with ER and Golgi, respectively, were clearly segregated away from lipid rafts (Fig. 3A). The high enrichment of annexin 2 in lipid raft was best appreciated when considering the total protein content in the fractions (Fig. 3B). To our knowledge, this is the first time that annexins have been approached by proteomics in lipid rafts.

In interpreting the proteotype of lipid rafts, the overall sparsity of proteins may indicate that the rafts at the time point of our analysis were at the "small" size stage (Gupta *et al.* 2006; Sezgin *et al.* 2017; Kusumi *et al.* 2020). Alternatively, it is possible that activation of receptors and induction of lipid raft merging may be intense in ESCs, in which case clustered rafts may detach from the plasma membrane in a vesicle like form and would not be detected in isolated rafts. This raft-dependent-endocytic pathway has been attracting attention over the past few years, across kingdoms
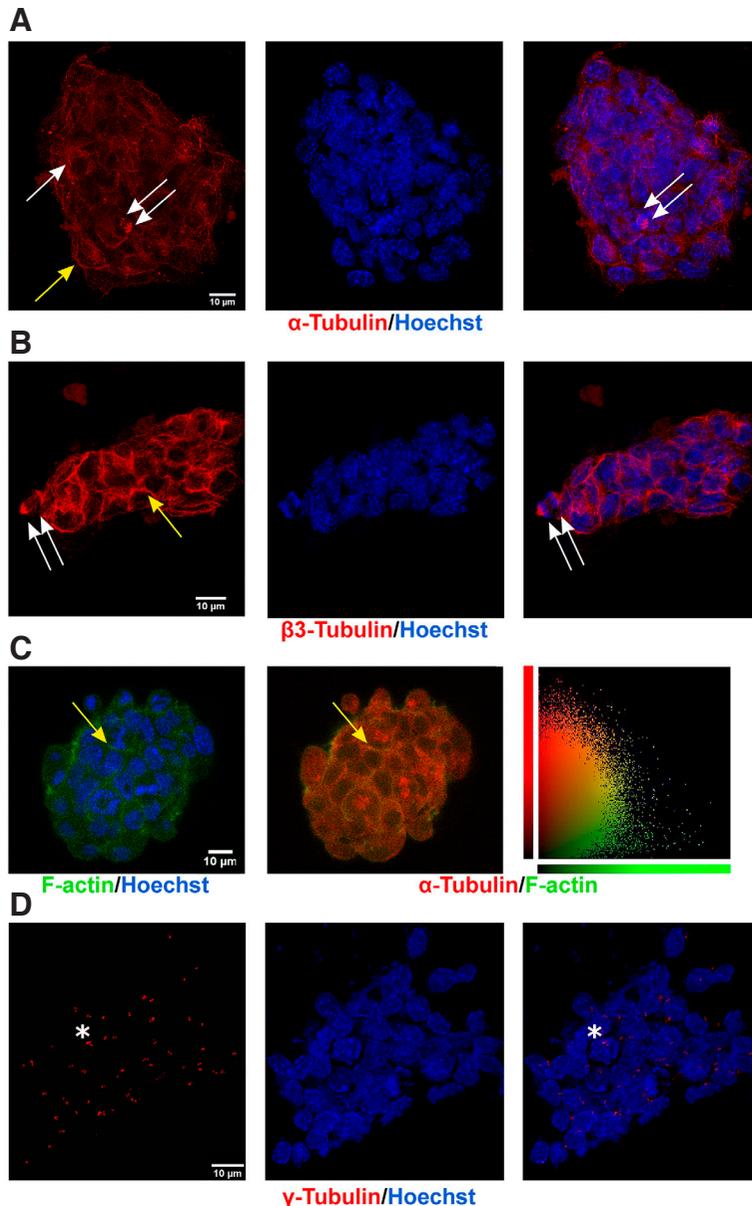

Fig. 4. Subcellular distributions of α-, β-, or γ-tubulin indicate actively dividing cells. ESCs, fixed as described in Methods, were incubated with indicated antibodies and with Hoechst 33258 for chromatin staining. **(A)** Representative projection confocal images (four focal planes of 0.7µm thickness, at 5 µm from the glass surface in the z-direction) of a single colony show α-tubulin on cytocolic (arrow), mitotic (double arrow), and peripheral (yellow arrow) MTs; last panel contains the merged α-tubulin and Hoechst images. **(B)** Typical confocal images (projection of four, 0.7 µm sections) of an ESC colony show staining of β3-tubulin in the periphery of the cells (yellow arrow) and in mitotic spindles (double arrow). **(C)** ESCs stained for α-tubulin (red) and F-actin (green) reveal a strong colocalization correlation (yellow arrows), readily seen in the scatter plot of the last column, which shows signal intensity of each voxel in the plane, as indicated in the label; PCC=0.288 ± 0.05. **(D)** Representative projection confocal images (four focal planes of 0.5 µm at 5 µm from the glass surface in the Z-direction) of an ESC colony stained with a γ-tubulin antibody decorates paranuclear centrosomes; adjacent staining indicates duplicated centrosomes (asterisks). bars =10 µm.

of life and even as a mechanism for entry of viruses, including corona-viruses (Li *et al.* 2007; Elkin *et al.* 2016; Wei *et al.* 2020). Endocytosis requires extensive actin and MT networks, to serve as tracks for intracellular vesicular trafficking. Indeed, use of colchicine and cytochalasin D, which disrupt microtubules and actin filaments, respectively, leads to redistribution of GPCR-Gs-AR in lipid rafts (Head *et al.,* 2006). Hence, our overall results postulate that lipid rafts in ESCs act in close association with the actin and tubulin cytoskeletons to support signal compartmentalization.

Lipid raft disruption by cholesterol depletion negates the mitogenic effect of prolactin

Many studies, including ours, have postulated that prolactin, a secreted polypeptide hormone, may act as a mitogen in several cell types, including precursor cells in the adult mouse hippocampus (Walker *et al.,* 2012), embryonic astrocytes (Mangoura *et al.,* 2000), pheochromocytoma and neuroblastoma cells (Cheng *et al.,* 2000), as well as many cancer stem cell types (Rouet *et al.,* 2010). Its growth factor-like actions were further supported by its role in improving the implantation potential in mouse blastocysts (Takeuchi *et al.,* 2017). Its specific receptor, PRLR, an archetype member of the cytokine receptor superfamily and a single-pass transmembrane protein, possesses no enzymatic activity. Thus, it exerts its signalling actions like LIF, through the activation of

JAK2, a cytokine-pre associated tyrosine kinase, and the transcription factors STAT1, 3, or 5 (Cheng *et al.,* 2000), and we detected its high expression in ESCs by immunocytochemistry (data not shown). Hence, we used prolactin as mitogen to test whether lipid raft integrity is required for completion of this signalling pathway.

First, we established that lipid raft integrity is necessary for propagation of mitogenic signalling pathways in ESCs. In order to disrupt lipid rafts, we used methyl-beta-cyclodextrin (MCD), a cyclic oligosaccharide that sequesters and thus depletes cells of cholesterol (Asimaki *et al.* 2011; Mangoura 2016; Karouzaki *et al.* 2019), for various exposure times and doses, and then recorded the number of mitoses. More specifically, 48 h after plating of ESCs on gelatin coverslips, cultures were treated either with vehicle, or with a low dose of 0.5mM MCD and were left to grow for another 1 or 2 days (Fig. 5A1, black and grey columns, respectively), and were then fixed and processed for mitotic figure analysis as described in Methods. When compared with their control counterparts, namely ESCs grown under identical conditions for 3 or 4 days but without MCD, a significant drop in the percentage of mitotic figures in the total population was evident. Therefore, long treatment with 0.5mM MCD significantly decreased mitotic activities in ESCs. When we used a higher dose of 5mM MCD and an expected >90 % of cellular cholesterol was depleted (Zidovetzki and Levitan, 2007), ESCs not only rarely displayed a mitotic figure, but most appeared to have apoptotic characteristics, such as cytoplasmic membrane blebbing and chromatin fragmentation (data not shown). Further establishing the importance of the plasma membrane cholesterol levels, when ESCs were "rescued" from MCD (i.e. cells were exposed to MCD by 1 h pulses every 24 h), the rates of mitotic figures per 3 or 4 days (stripped columns, respectively) did not differ statistically from controls, as is also clearly seen in typical confocal images in Fig. 5A2.

As shown in Fig. 5A1, the rate of mitoses under normal culture conditions falls significantly between day 3 and 4 in culture. Therefore, we chose to treat cells with prolactin (100nM) on the 3rd day and assess numbers of mitotic figures in the presence or absence of 0.5mM MCD. Prolactin acted as a strong mitogen in ESCs, with a significant 4-fold rise in the numbers of mitoses in the total cell populations (Fig. 5B). This mitogenic effect
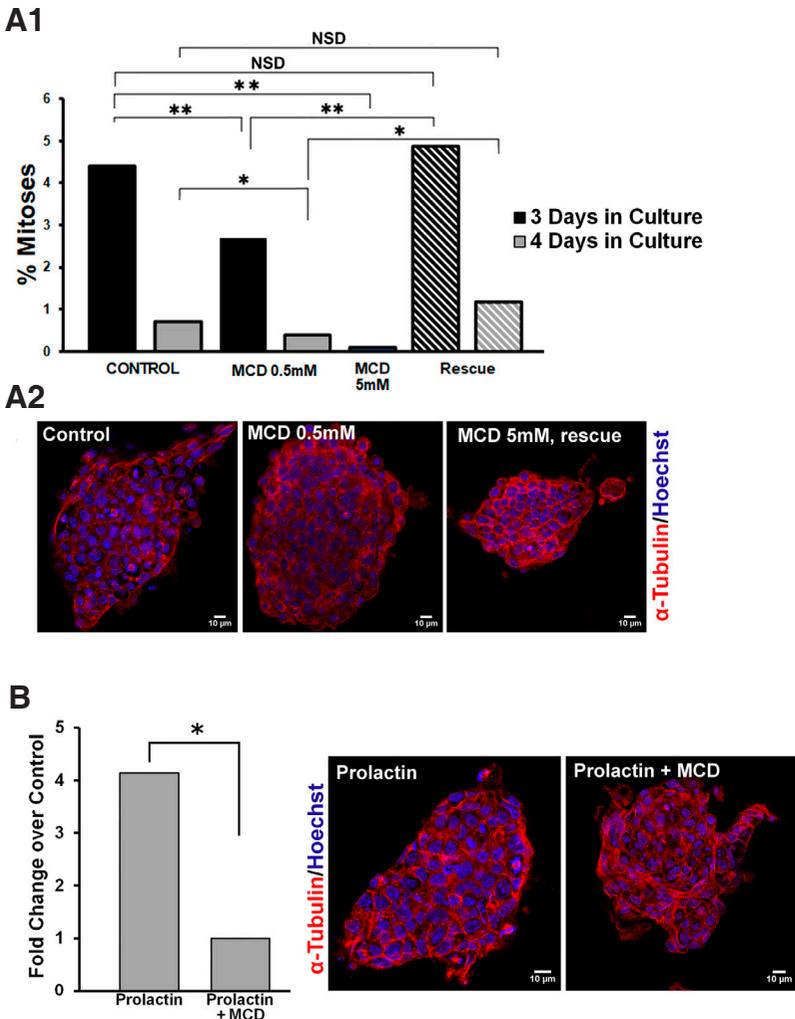
A1

A2

B

Fig. 5. Prolactin significantly increases the mitotic index in ESCs and requires intact lipid rafts. (A1) Lipid raft integrity is required for mitotic expansion of ESCs. ESCs cultured 3 or 4 days in total, black and grey columns, respectively, were treated continuously with vehicle, 0.5mM MCD, or 5mM MCD, or in pulses of 1 hour MCD for the same time periods (hatched columns); upon completion of treatments, the ratio between the number of cells in mitosis and the total number of cells (mitotic index) was calculated in samples immunostained for α-tubulin (red) and co-stained with Hoechst for chromatin (blue). **(A2)** Representative, single focal planes of samples used in A1. **(B)** Lipid raft integrity controls the mitogenic effect of prolactin. ESCs were treated after 3 days in culture with 100nM prolactin with or without 0.5 mM MCD, and the resulting mitotic indeces show that the high mitogenic effect of prolactin is abolished by concurrent treatment with MCD. Panels show representative, single focal planes of samples used for these calculations. * p <0.05, ** p ≤0.01, *** p ≤ 0.001, NSD, no significant difference.

was curtailed significantly by MCD, also clearly visible in confocal images of cells stained for alpha tubulin and Hoechst. Therefore, while the association of the prolactin receptor subunits with the lipid rafts needs to be further investigated, similarly, our results suggest that lipid rafts are signalling platforms for the prolactin-induced mitogenic pathway in ESCs. Our studies highlight the critical role of well-organized lipid rafts in the intracellular transduction of signals from the PRLR, a receptor that is widely expressed in stem cells and which gains multifold expression prior to blastulation of human embryos (Ezoe *et al.*, 2021). Moreover, blastocyst outgrowth is also significantly increased in human embryos cultured in the presence of PRL, further strengthening the suggestion that manipulation of lipid rafts in stem cells may be a target for controlling their expansion, a field of considerable interest for cancer stem cells (Rouet *et al.*, 2010)).

Concluding Remark

We have further defined and expanded the mouse E14 stem cell proteome and have highlighted in particular its close association with the major cytoskeleton assemblies of actins and tubulins. Apparently, ESCs have strikingly developed cytoskeletons, unexpected for non-polarized cells, giving rise to speculation that the close association between the signalling platforms of the lipid rafts and the actin and tubulin systems may prevent cell polarization. As ESCs grow exclusively in situ as multi-cell colonies with their cell motion practically restricted to cytokinesis, this complex lipid raft- cytoskeleton assembly may also regulate lipid raft-dependent signalling for self-renewal and growth, i.e. for symmetric cell division.

Materials and Methods

Antibodies

Goat polyclonal antibody to calnexin was purchased from Santa Cruz Biotechnology (SCBT). Mouse monoclonal antibodies to actin, coatomer protein beta (β-COP), and to α- and β-tubulin from Sigma-Aldrich; to tubulin β3 (TUJ1) from Covance, to γ-tubulin from Abcam, and to flotilin-1 from SCBT. Rabbit polyclonal antibodies to annexin 2 and a rat monoclonal antibody to H-Ras were also from SCBT.

Chemicals

Methyl-beta-cyclodextrin (MCD) was purchased from Sigma-Aldrich, and prolactin was kindly provided by the National Hormone and Pituitary Program, National Institutes of Health. The source of all chemicals and reagents used is indicated when first described; standard chemicals were from Sigma.

ES Cell culture

The E14 ESC line (Chambers *et al.*, 2003) was maintained on 0.1% gelatin-coated dishes without a feeder layer of fibroblasts (Bibel *et al.*, 2004) in high-glucose (10%) DMEM, supplemented with 15% stem cell-certified fetal bovine serum, FBS (Biowest), 6mM L-glutamine, non-essential amino acids (Invitrogen), 1000 units/ml LIF (Millipore), and 0.1mM β-Mercaptoethanol (Acros Organics), at 37˚C and 10% CO_2. The W4 cell line (a generous gift from Dr. A. Klinakis, BRFAA), used to verify lipid rafts protein expression, was cultured under identical conditions.

ES Cell culture treatments

Treatments on ESCs were done routinely after two days in culture, using 0.5mM or 5mM of the cholesterol-sequestering agent Methyl-β-cyclodextrin (MCD) and/or 100nM Prolactin in the presence of 3% FBS and LIF.

Membrane subfractionation

Lipid rafts were isolated using a detergent-free method that preserves the native composition of these lipid-protein micro-domains, as previously reported (Macdonald and Pike 2005; Asimaki *et al.* 2011; Karouzaki *et al.* 2019). In brief, $4x10^6$ cells were resuspended in base buffer consisting of 20 mM Tris-HCl pH 7.8, 250 mM sucrose, 1 mM calcium chloride, 1 mM magnesium chloride, and protease and phosphatase inhibitors and then lysed by repeated passage through a 23 G needle. Lysates were centrifuged at 1000 x *g* for 10 min, and the resulting postnuclear supernatant was collected and transferred to a separate tube. An equal volume of base buffer containing 50% OptiPrep was added, and all were placed in the bottom of a 12 ml centrifuge tube. A 10 ml gradient of 0% to 20% OptiPrep in base buffer was poured on top and gradients were centrifuged for 90 min at 52,000 x *g* using a TH-641 rotor in a Sorval Discovery 90 ultracentrifuge. Gradients were fractionated into fourteen 0.7 ml fractions. As the total protein content was low (Asimaki *et al.* 2011; Karouzaki *et al.* 2019), the first five fractions, representing lipid rafts, were pooled for proteomics analysis, while for Western blotting analysis they were run individually. Fractions or lysates were boiled in Laemmli buffer and the distribution of the various proteins was assessed with 1DE and Western blotting or 2DE and MS-MS.

Two-dimension electrophoresis (DE) of gels and peptide mass fingerprint (PMF)

2D gel electrophoresis was performed as previously described (Anagnostopoulos *et al.,* 2011). Briefly, 1 mg of protein was applied on 18cm immobilized pH 3-10 non-linear gradient strips (Bio-Rad) and isoelectric focusing was carried out with at 3 V/min for 18 h; the second-dimensional separation was performed in 12% SDS-polyacrylamide gels at well-defined conditions. Proteins were visualized with colloidal Coomassie Brilliant Blue and scanned with a GS-800 densitometer.

Peptide analysis and protein identification were performed as described (Berndt *et al.,* 1999). Spots were automatically detected by Melanie 4.02 software (GeneBio) on the Coomassie-stained gels, excised by the Proteiner SPII (Bruker Daltonics), destained with 30% acetonitrile in 50 mM ammonium bicarbonate and dried in a speed vacuum concentrator. Each dried gel piece was rehydrated with 5µl of 1 mM ammonium bicarbonate containing 50 ng trypsin, overnight at room temperature. Twenty microliters of 50% acetonitrile - 0.1% trifluoroacetic acid were added for 15 min with constant shaking. The resulting peptide mixture (1 µl) was simultaneously applied with 1 µl of matrix solution, consisting of 0.8% α-cyano-4-hydroxycinnamic, standard peptides des-Arg-bradykinin (904.46 kD), and adrenocorticotropic hormone fragment 18-39 (2465.19 kD). Samples were analyzed for PMF with Matrix-Assisted Laser Desorption-Mass Spectrometry (MALDI-MS) in a time-of-flight mass spectrometer (Ultraflex II). Laser shots (n = 400) at intensity between 40% and 60% were collected and summarized, and peak lists were created using the Bruker FlexControl v2.2 and Flexanalysis v2.2 software. Each

spectrum was interpreted with Mascot Software v2.0 (Matrix Sciences). For peptide identification, the monoisotopic masses were used and a mass tolerance of 0.0025% (25ppm) was allowed. All extraneous peaks, such as trypsin autodigests, matrix, and keratin peaks, were not considered for the protein search. Peptide masses were compared with the theoretical peptide masses of all available proteins from rodents in the SWISS-PROT and TREmBL databases, which are updated bimonthly. The probability score identified by the software was used as the criterion for identification.

Western blotting (WB)

WB analysis was essentially performed as previously described (e.g. Karouzaki *et al.,* 2019). Lipid raft fractions were analyzed as equal volumes (50 μl) of OptiPrep fractions in Laemmli buffer per well of a polyacrylamide gel by electrophoresis (SDS-PAGE), after which proteins were transferred onto nitrocellulose membranes and probed with primary antibodies. Immunoreactivity was visualized by incubation with the appropriate species-specific antibody, conjugated to horseradish peroxidase (HRP) or alkaline phosphatase (AP), and ECL chemiluminescence or AP substrate colorimetric reaction, respectively. Exposed films or stained membranes were then scanned and exported as TIFF files. All experiments were repeated at least four times with similar results.

Immunocytochemistry

For immunocytodetection of proteins, cells were treated with the protein crosslinker disuccinimidyl propionate (DSP), in order to best preserve cytoskeletal assemblies (Leondaritis *et al.* 2009; Peta *et al.* 2020), extracted with 0.1% Triton, and then fixed with 4% paraformaldehyde (PFA). After blocking of non-specific sites with serum from the appropriate species, cells were first incubated with primary antibodies and then with the appropriate secondary antibodies conjugated to rhodamine (red), while chromatin was stained with Hoechst 33258 (blue) (Pharmingen). F-actin was visualized with Oregon Green 488 phalloidin (ThermoFisher) or Alexa Fluor 633 phalloidin (Invitrogen) staining. Stained cells were imaged with a Leica TCS SPT laser inverted scanning confocal microscope, equipped with two ion lasers through a 63X HC PC APO CS objectives and Tandem Scanner (Leica Microsystems, Biological imaging facility, BRFAA), and z-stacks with 0.34μm-thick z optical sections. All Images were analyzed using LAS-AF software, deconvolved using Huygens Essential (SVI) and saved in a TIFF format.

Quantification of mitotic cells

The analysis of the mitotic cells was performed with the Cell Counter plugin of the ImageJ software. The area of the nucleus was defined from Hoechst fluorescence, and the mitotic cells from Hoechst fluorescence and β-tubulin immunostaining of confocal images, captured in z-stacks with 0.34 μm-thick z optical sections. All mitotic cells (in prophase, metaphase, anaphase or telophase) and nuclear foci were marked with a coloured square, given a unique number, and added to a tally sheet; results were exported as an Excel file for statistical analysis. The total percentage of mitoses that were calculated as mitotic figures over total numbers of nuclei from 2-3000 cells for each condition (Fig. 5).

Colocalization Analysis

Colocalization analysis of α-tubulin and F-actin was performed with the Volocity® 6.1.2 software, using the Costes Pearson Correlation Coefficient (PCC) algorithm as described in (Peta *et al.,* 2020). Regions of interest (ROI) were drawn manually around the cells (100 cells from 3 different experiments) and the extent of overlap between fluorophores were calculated within a range of 1, if a perfect positive correlation, to -1, if a perfect but inverse correlation; 0 represents a random distribution. PCC means were automatically calculated from the generated scattered plots and exported for every single plane in an excel file.

Semi-quantitative PCR

SemiQ-PCR was performed as previously described (Mangoura *et al.* 2006; McLaughlin *et al.* 2006). Briefly, total RNA isolated from embryonic stem cell cultures, using the Trizol reagent (Ambion), was reverse transcribed (1 μg per reaction) with SuperScript II reverse transcriptase (Invitrogen), and 1μL of the formed cDNA was amplified with Taq DNA Polymerase (New England Biolabs), in the presence of gene-specific primers for: Nanog-P1, 5' GCGGACTGTGT-GTTCTCTCAGGC 3'; Nanog-P2, 5' TTCCAGATCCGTTCACCAGATAG 3'; Oct4-P1, 5' GGCGTTCGCTTTGGAAAGGTGTTC 3'; Oct4-P2, 5' CTCGAACCACATCCTTCTCT 3'; and, used as control, β-actin-P1, 5' TGACGGGGTCACCCACACTGTGCCCATCTA 3'; β-actin-P2, 5' CTAGAAGCAGCGGTGGAGGATGGATGGA GGG 3'. Amplification conditions, for Nanog and Oct4, were an initial denaturation step of 5 min at 94°C followed by 33-40 cycles (linear region) at 94°C for 1 min, at 60° C for 30 sec, at 72° C for 30 sec, followed by 10 min at 72°C. For β-actin, the corresponding amplification steps were: 94° C, 5 min; 94° C, 1 min; 56° C, 30 sec; 72° C, 1 min; final extension 72° C for 5 min. PCR products, separated in appropriate for each product percentage agarose gels, were stained with ethidium bromide and photographed under UV light using the Dolphin-Doc Pro system.

Statistics

All experiments were performed three to ten times with similar results, and numerical data were analyzed by ANOVA, using the SPSS software (IBM) and a set statistical significance level (p) of 0.05. Graphs were generated with the same software, and images were organized using Adobe Photoshop.

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