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Involvement of the transcription factor FoxM1 in contact inhibition

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ABSTRACT

Contact inhibition is a crucial mechanism regulating proliferation *in vitro* and *in vivo*. Although it is generally accepted that contact inhibition plays a pivotal role in maintaining tissue homeostasis, the molecular mechanisms of contact inhibition are still not fully understood. FoxM1 is known as a proliferation-associated transcription factor and is upregulated in many cancer types. Vice versa, anti-proliferative signals, such as TGF-β and differentiation signals decrease FoxM1 expression. Here we investigated the role of FoxM1 in contact inhibition in fibroblasts. We show that protein expression of FoxM1 is severely and rapidly downregulated upon contact inhibition, probably by inhibition of ERK activity, which then leads to decreased expression of cyclin A and polo-like kinase 1. Vice versa, ectopic expression of FoxM1 prevents the decrease in cyclin A and polo-like kinase 1 and causes a two-fold increase in saturation density indicating loss of contact inhibition. Hence, we show that downregulation of FoxM1 is required for contact inhibition by regulating expression of cyclin A and polo-like kinase 1.

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1. Introduction

Cell–cell contact is known to be a critical regulator of cellular proliferation, differentiation and motility. Inhibition of proliferation by cell–cell contact is generally referred to as contact-dependent inhibition of growth or contact inhibition [1]. *In vitro*, non-transformed cells are arrested in G0/G1-phase at a critical cell density forming a confluent monolayer. In adult tissues, contact inhibition is thought to be continuously active, playing a critical role in the repression of somatic cell proliferation and probably organ size control [2]. The importance of contact inhibition for tissue homeostasis is demonstrated, for instance, by the fact that hypersensitivity to contact inhibition in the naked mole-rat provides cancer resistance in these animals [3]. Vice versa, release from contact inhibition *in vivo* and *in vitro* is associated with abnormal cellular proliferation and tumorigenesis [4]. In line, transformed cells are characterized by a loss of contact inhibition which is manifested by a higher saturation density and the emergence of multi-layered foci *in vitro*. Despite its importance for cell cycle control, knowledge about the signaling cascade mediating contact inhibition is still scarce [5].

One central regulator for G1 cell cycle progression and transition into S-phase is the ERK-Cyclin D/Cdk4-pRB-pathway [6]. Activation of ERK, for instance by growth factors, leads to expression of cyclin D1, which in association with Cdk4 (or Cdk6) phosphorylates the retinoblastoma protein (pRB), the gate keeper of G1-S-transition [7]. Downstream, pRB is phosphorylated by the cyclin E/Cdk2 complex thereby allowing dissociation from the transcription factor EF2 which then activates transcription of S-phase specific genes, such as cyclin A [6,8]. Upon contact inhibition in fibroblasts, phosphorylation of pRB is blocked by inhibition of ERK activity, subsequent downregulation of cyclin D1 as well as upregulation of the cdk4 inhibitor p16 and the cdk2 inhibitor p27 [9–13].

Gene expression studies comparing confluent versus proliferating cultures of mouse fibroblasts revealed differential expression of genes which are involved e.g. in proliferation, signal transduction, transcriptional regulation, cell adhesion and communication [12]. One interesting observation was downregulation of the transcription factor FoxM1. FoxM1 has been described as Trident, WIN (winged helix from INS-1 cells), FKHL 16 (forkhead drosophila homolog-like 16), MPP2 (MPM2-reactive phosphoprotein2, M-phase phosphoprotein 2), and HFH-11 [HNF-3(hepatocyte nuclear factor 3)/forkhead homolog 11] reviewed in [14]. It belongs to the forkhead/winged helix transcription factors and is known to be a typical proliferation-associated transcription factor which is downregulated upon anti-proliferative signals, such as serum-depletion, differentiation, aging or TGF-β [14]. In adult tissues, expression of FoxM1 is limited to self-renewing epithelia, e.g. small intestine or colon, or organs with a large fraction of proliferating cells, such as thymus or testis [14]. In non-proliferating tissues, such as lung or liver, expression is very low. However, expression is re-induced during regenerative proliferation, for instance due to injury or partial hepatectomy [14]. In line, upregulation of FoxM1 has been described in several cancer types and FoxM1 is known to be one of the few genes which are upregulated during early cancer development [14,15]. Its dominant role during

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proliferation is demonstrated by regulation of genes being involved in G1/S and G2/M transition, such as Skp2 and Cks, which are subunits of the Skp1/cullin/F-box protein (SCF) complex regulating degradation of the cyclin-dependent kinase inhibitors p21 and p27, as well as expression of cyclin D1, Cdc25B, cyclin A, cyclin B, Aurora B kinase, survivin, polo-like kinase 1 (Plk1) and centromere protein A (CENPA), CENPB, and CENPF [14]. Interestingly, to date nothing was known about its regulation upon contact inhibition. Here we show that downregulation of FoxM1 is crucial for contact inhibition. In confluent cultures, expression of FoxM1 is diminished leading to a decrease in cyclin A and Plk1. Vice versa, ectopic expression of FoxM1 causes a loss of contact inhibition by preventing downregulation of these proteins. Time course analysis revealed early decrease in FoxM1 upon contact inhibition, very likely as a result of decreased ERK activity. We provide a model in which the decrease in ERK activity upon contact inhibition not only blocks the cyclin D/Cdk4-pRB-pathway, but additionally induces downregulation of FoxM1 which is essential to prevent G1/S transition and further cell cycle progression.

2. Materials and methods

2.1. Cell culture

Non-transformed NIH3T3 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (PAA) supplemented with 10% fetal calf serum (FCS) (PAA), 4 mM glutamine, penicillin and streptomycin (100 U/ml).

2.2. Flow cytometry

Cells were either seeded to a low density of $1.4 \times 10^4/cm^2$ (60% confluence) or to a high density of $1.8 \times 10^5/cm^2$ (100% confluence) and cultured for 24 and 48 h. Cells were trypsinized and washed twice with phosphate-buffered saline (PBS). $1–2 \times 10^6$ cells were vortexed in 200 μl of PBS and fixed with 2 ml of ice-cold 70% ethanol for 30 min at 4 °C. Cells were then permeabilized by incubation with 1 ml of 0.2% Tween 20/PBS for 15 min at 37 °C. Cells were resuspended in 2% FCS/PBS in the presence of RNAse A (11.25 kU/sample) and incubated with propidium iodide (50 μg/sample, Applichem) for 30 min at room temperature in the dark. Finally, the cells were resuspended in 800 μl of PBS and flow cytometric analysis was performed by a FACSCalibur (Becton Dickinson).

2.3. Western blot

Cells were either seeded to a low density of $1.4 \times 10^4/cm^2$ (60% confluence) or to a high density of $1.8 \times 10^5/cm^2$ (100% confluence), cultured and treated as described in the figure legend. Total cell extracts were prepared by lysing the cells in hot Laemmli sample buffer and protein concentration was determined according to Smith et al. [16]. Equal amounts of protein (20–50 μg protein/lane) were separated by SDS-PAGE (7.5–12.5%) and electroblotted overnight onto Immobilon membrane (Millipore). The membranes were blocked for 1 h with 5% low-fat milk-powder in TBS (50 mM Tris–HCl, pH 7.5, 150 mM NaCl) containing 0.05% Tween 20 and then incubated for 1.5 h at room temperature with anti-FoxM1-, anti-cyclin A-, anti-Plk-1-antibody (1:1000, Santa Cruz) or anti-phospho-ERK1/2 (1:1000, Cell Signaling) followed by incubation with horseradish-peroxidase-conjugated secondary antibody and ECL-detection according to the manufacturer's instructions. To control equal loading, the blots were stripped and reprobed with anti-ERK2- or anti-ERK1/2-antibody (1:2000, Santa Cruz).

2.4. Transient transfection of siRNA

NIH3T3 cells were transfected with FoxM1 siRNA (MWG Biotech AG) or control siRNA (Sigma), respectively, using RNAiMAX (Invitrogen). In 24-well-plates, 28 pmol FoxM1siRNA or control siRNA, respectively, was diluted in 100 μl of Opti-MEM I reduced medium. 1.4 μl of RNAiMAX reagent were added and incubated for 20 min at room temperature. 2×10^4 NIH3T3 cells were diluted in 500 μl of DMEM/10% FCS without antibiotics, added to each well (final concentration of siRNA 47 nM) and incubated for another 5 h at 37 °C. Medium was changed to DMEM/10% FCS containing antibiotics. Cells were cultured for another 48 h at 37 °C. FoxM1 siRNA: GGACCACUUCCCUUACUU UTT (sense), AAAGUAAGGGAAGUGGUCC (antisense).

2.5. RT-PCR

RNA was isolated by RNeasy Mini Kit (Qiagen) and reverse-transcribed by Advantage RT-for-PCR Kit (Clontech). Primers, PCR conditions and fragment sizes are listed in Supplementary Table 1.

2.6. Stable transfection with pCMV-FoxM1

NIH3T3 cells were stably transfected with pCMV-Foxm1b (generously provided by Pradip Raychaudhuri, Illinois) [17] or empty vector and pcDNA6/TR (Invitrogen) carrying the blasticidin resistence gene using Lipofectamine (Invitrogen). NIH3T3 cells were grown in DMEM/10% FCS to 80% confluence (100 mm culture dishes). Before transfection, medium was replaced by 3.2 ml DMEM without FCS and without antibiotics. For transfection, 7 μg of pCMV-FoxM1 and 1.5 μg of pcDNA6/TR were diluted in 800 μl of Opti-MEM I reduced medium and incubated with 50 μl of Lipofectamine, also diluted to 800 μl of Opti-MEM I reduced medium, for 30 min at room temperature. 1.6 ml of Opti-MEM I reduced medium was added to the complex solution (total volume 3.2 ml) which was then added to the cells. After 5 h incubation at 37 °C, 6.4 ml of DMEM/20% FCS (no antibiotics) was added. After another 24 h, cells were passaged in DMEM/10% FCS, antibiotics and 3 μg/ml blasticidin (Invitrogen). After 8–10 days colonies were picked.

3. Results

Cultures of NIH3T3 cells undergo contact inhibition. When they are seeded to confluence, about 75% of the cells are arrested in G0/G1-phase after 24 h, and more than 90% are arrested in G0/G1 after 48 h, respectively (Fig. 1A, [12,13]). Simultaneously, we detected a strong decrease in the expression of the transcription factor FoxM1 and of its downstream-targets cyclin A and Plk1 (Fig. 1B). Downregulation of FoxM1 upon contact inhibition was not restricted to NIH3T3 cells, but was observed in all cell lines tested including rat liver oval cells (WB-F344), mouse C3H10T1/2 fibroblasts and human colon epithelial cells (Caco-2) indicating that FoxM1 levels generally decrease upon contact inhibition (Fig. 1C and unpublished observation).

To investigate whether the observed downregulation of cyclin A and Plk1 was caused by the decrease in FoxM1 expression, we knocked-down FoxM1 by transient transfection of siRNA targeted against FoxM1 and performed RT-PCR and Western blot analysis after 48 h. Fig. 2A and B clearly shows that downregulation of FoxM1 results in decreased mRNA and protein expression of cyclin A and Plk1. In line with the work of others reviewed in [14], we also observed upregulation of the cdk inhibitor p27 (data not shown). As a result, proliferation was blocked which was demon-

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