Human Embryonic Stem Cells: Stable or Vulnerable?

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Human embryonic stem cells (hESC) are immature cells derived from human blastocyst-stage embryos that can be indefinitely propagated in culture and can also be induced to differentiate into all mature cell types with special function. These two abilities, to self-renew and to differentiate, make hESC attractive tool for cell replacement therapy, drug development, and toxicology. One of the key criteria that have to be met before hESC are used for therapy is a certainty that the cells are void of any risk of unpredictable behavior. Among such behaviors, deregulated growth of hESC derivatives upon their transplantation to the patient is the most hazardous one. Several studies have recently reported accumulation of various types of alterations to DNA of hESC that have been propagated in culture for prolonged periods of time. What types of stress induces such damage, contribution of hESC line genotype, what are the biological outcomes of such damage, and many other questions remain to be answered.

We have recently begun addressing some of the issues related to the genomic instability of hESC. As numerical and structural abnormalities of centrosomes contribute to chromosomal instability in many cancers, we have assessed the metabolism of centrosomes in hESC. We have found in several independent cells lines that undifferentiated hESC are typical by unusually high frequency of mitoses with the number of centrosomes exceeding two. Labeling for gamma tubulin demonstrated that most of these supernumerary centrosomes in fact participated in formation of multipolar spindles. Importantly, although centrosome amplifications occur in high frequency (10-30%) in hESC in early to mid passages they become suppressed in hESC in later passages (less then 5%). We have also found that culture conditions, mainly ability of hESC to appropriately adhere to the culture substratum, influence the overamplification of centrosomes. Several molecules have been previously shown to drive amplification of centrosomes in normal cell cycle. We have found that at least two of them, Aurora A and CDK2, are overabundant and highly active in undifferentiated hESC and may thus contribute to the observed abnormalities. This is supported by the fact that chemical inhibition of CDK2 in hESC reduces the percentage of mitoses with supernumerary centrosomes. Both the integrity and number of centrosomes seem to be monitored by signaling pathways that employ established molecules involved in sensing and executing response to DNA damage. Here we show that at least some components of such pathways are developed and operative in hESC. Upon DNA damage induced by UVC-irradiation damage, hESC accumulate p53 protein that is capable of transactivation of its target genes (p21, GADD45, mdm2). UVC-irradiated hESC also phosphorylate Chk2, degrade their CDC25A phosphatase, and, according to at what stage of cell cycle hESC are exposed to DNA damaging insult, delay their cell cycle in G1 or G2/M phase.

In summary, we demonstrate that although undifferentiated hESC possess some protective

mechanisms to maintain its DNA pristine, they still develop conditions that are favorable to generation of genetic abnormalities. We propose that understanding of mechanisms involved in harboring early alterations to genome of originally genetically normal hESC, and their link to the changes in their phenotype in particular, will shed some light on initial steps of carcinogenesis.

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ANALÝZA EFEKTU INHIBICE CYKLIN DEPENDENTNÍCH KINÁZ NA DIFERENCIACI LIDSKÝCH EMBRYONÁLNÍCH KMENOVÝCH BUNĚK

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Cyklín dependentné kinázy (CDK), cyklíny a inhibítory cyklín dependentných kináz predstavujú molekuly, ktorých primárnou funkciou je regulácia priechodu bunky bunkovým cyklom. Súčasné výsledky získané nie len v našom laboratóriu naznačujú, že tieto molekuly sa zúčastňujú aj regulácie diferenciácie a to spôsobom, ktorý nemusí priamo súvisieť s reguláciou proliferácie.

Ľudské embryonálne kmeňové bunky (hESC – human embryonic *s*tem cells) sú pre svoje jedinečné vlastnosti v súčasnosti využívané pre výskum ranného embryonálneho vývoja, procesu diferenciácie buniek a skrývajú najmä potenciál pre využitie v klinickej praxi. Mechanizmy, ktoré sa uplatňujú v riadení proliferácie nediferencovaných buniek a vstupu buniek do diferenciačných pochodov zatiaľ nie sú presne známe. Výrazný rozdiel medzi ES bunkami a somatickými bunkami predstavuje regulácia a doba trvania bunkového cyklu. ES bunky majú cyklus výrazne rýchlejší, majú redukovanú dĺžku G1 fázy cyklu a vykazujú prítomnosť iba hyperfosforylovanej formy pRB proteínu. Môže to znamenať, že bunky nemajú funkčný reštrikčný bod v G1 fáze a sú tak necitlivé na určité regulačné signály. Inhibícia cyklu v tejto fáze by mohla poskytnúť bunkám dostatok priestoru na prijatie mitogénnych signálov a indukovať či aspoň „uľahčiť“ tak u nich prechod do diferenciačnej dráhy.

Prezentovaná práca sa zaoberá analýzou efektu inhibície cyklín dependentných kináz špecifickými syntetickými inhibítormi (pripravenými v laboratóriu Prof. Strnada, UP v Olomouci) na diferenciáciu ľudských embryonálnych kmeňových buniek *in vitro*. Zo získaných dát na ľudských ES bunkách v nízkej pasáži vyplýva, že aspoň jeden z analyzovaných inhibítorov (Olomoucin II – OC II), pridávaný do bežného kultivačného média v nízkych koncentráciách ($\leq$2 μM), spôsobuje morfologické (diferenciačné?) zmeny, ktoré sú odlišné od zmien typických pre spontánne diferencujúce bunky (kultivácia bez OC II). Analýza bunkového cyklu ukázala, že hES bunky ošetrené OC II sa kumulujú v G2/M fáze. Je teda pravdepodobné, že OC II diferenciáciu indukuje, nie však mechanizmom asociovaným s predĺžením G1 fázy bunkového cyklu. Pri vyšších koncentráciách (>2 μM) OC II pôsobí na bunky cytotoxicky a indukuje u nich bunkovú smrť.

Reakcia na prítomnosť OC II je odlišná u hES buniek dlhodobo propagovaných *in vitro* (bunky vo vysokej pasáži), ktoré sú adaptované na kultivačné podmienky. Tieto bunky prežívajú pri vyšších koncentráciách OC II (4 μM) než bunky v nízkych pasážach. Zároveň, v porovnaní s bunkami v nízkej pasáži, rastie u adaptovaných buniek aj intenzita diferenciačnej reakcie na prítomnosť (a regulačný efekt) OC II. Tieto zmeny bezpochyby predstavujú efekt doposiaľ nedostatočne poznaných molekulárnych prestavieb, ku ktorým v priebehu dlhodobej propágacie hES buniek *in vitro* dochádza.

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REACTION OF HUMAN EMBRYONIC STEM CELLS TO UVC-INDUCED DNA DAMAGE

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Human embryonic stem cells (hESC) are pluripotent cells with the capability to maintain their undifferentiated state when propagated in culture. Their ability to self-renew and to differentiate into various cell types of adult organism makes these cells attractive tool for cell replacement therapy, drug development and toxicology. Potential application of hESC in cell therapies requires maintaining the cells genetically pristine during prolonged propagation *in vitro*. However, during propagation *in vitro*, hESC are exposed to various types of stress, which can cause alternations in their DNA. Such alternations may be equivalent to those commonly found in cancer cells.

The aim of this study was to determine whether hESC contain molecular components involved in ATM/ATR signaling pathways and also whether their G1/S and/or G2/M checkpoints are developed and fully functional. As the regulation of ATM/ATR pathways is dependent on the type and the extent of DNA damage, here we studied the effect of different doses of UVC irradiation on line CCTL14 of hESC. We found that after irradiation hESC accumulate in G2 phase of cell cycle, suggesting that they do not activate G1/S phase checkpoint. Cdc25A phosphatase is crucial to both ATM and ATR pathways. We found that Cdc25A phosphatase becomes degraded at doses of UVC $\geq$ 1 J/m^2. Moreover, western blot analysis showed that hESC at doses of UVC $\geq$ 1 J/m^2 undergo apoptosis and activate stress pathways involving p38 and p53, which are functionally connected with ATM/ATR. Surprisingly, hESC maintained in culture for high number of passages require much higher dose of UVC irradiation (~5 J/m^2) to initiate the same molecular response. It is of note that such increased resistance to UVC-induced DNA damage is often observed in cancer cells.

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Human embryonic stem cells can activate checkpoint control pathways

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Human embryonic stem cells (hESC) are pluripotent cells with the capability to maintain their undifferentiated state when propagated in culture. Their ability to self-renew and to differentiate into various cell types of adult organism makes these cells attractive tool for cell replacement therapy, drug development and toxicology. Potential application of hESC in cell therapies requires maintaining the cells genetically pristine during prolonged propagation *in vitro*. However, during propagation *in vitro*, hESC are exposed to various types of stress, which can cause alternations to their DNA. Such alternations may be equivalent to those commonly found in cancer cells.

The aim of this study was to determine whether hESC contain molecular components involved in ATM/ATR signaling pathways and also whether their G1/S and/or G2/M checkpoints are developed and fully functional. As the regulation of ATM/ATR pathways is dependent on the type and the extent of DNA damage, here we studied the effect of different doses of UVC irradiation on hESC. We found that DNA damage in early G1 phase causes hESC to arrest their cycle in G1/S phase, suggesting the presence of G1/S checkpoint pathway. CDK2 and its upstream regulatory molecule Cdc25A are the crucial molecules that drive cell through G1/S transition. We found that Cdc25A phosphatase becomes degraded and activity of CDK2 is lowered at doses of UVC $\geq$ 1.5 J/m^2. Moreover, western blot analysis showed that hESC at doses of UVC $\geq$ 1 J/m^2 undergo apoptosis and activate stress pathways involving p38 and p53, which are functionally connected with ATM/ATR. We found that p53 is localized in the nucleus and at centrosomes in the absence of DNA damage. We also found that p53 can activate its target gene p21 in response to UVC-induced DNA damage.

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How do hESC sense and respond to damage to their DNA?

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Curiculum Vitae

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Chemical Inhibition of CDKs Drives Human Embryonic Stem Cells Into Differentiation

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Human embryonic stem cells (hESC) are lines of pluripotent cells with the ability to indefinitely self-renew and capacity to differentiate into almost all somatic cell types. Thanks to these characteristics, hESC create new opportunities to both basic research and clinical medicine. Although mechanisms that drive hESC into differentiation are not yet fully understood, it is hypothesized that fast proliferation and limited length of G1 phase contribute to the maintenance of hESC in undifferentiated status. Cyclin-dependent kinsases (CDK), cyclins and inhibitors of CDKs are key regulators of cell cycle progression. The aim of this study was to analyze the effect of inhibition of CDKs with synthetic inhibitors (synthesized in the laboratory of Growth Regulators at Palacky University in Olomouc) on differentiation of hESC (line CCTL -14). The data show that exposure of hESC maintaned under standard nondifferentiating culture conditions to synthetic inhibitor 2,6,9-trisubstituted purine - Olomoucine II (OC II) promotes differentiation changes in such cells. Notably, the changes induced by OC II were different from those observed in spontaneously differentiating hESC. Flow-cytometric analysis of the cell cycle showed that after 5 and/or 24 hour treatment with OC II hESC accumulate not only at G1 but also at G2/M phase of their cell cycle. Correspondingly, immunoprecipitation of CDKs followed by in vitro measurement of their activity have shown that OC II inhibits both CDK 2 and CDK 1. Prolonged cultivation of hESC with OC II in standard culture media have lead to developement of expression of neuro-specific markers Sox1, Neuro D1, and Nestin as determined by RT-PCR and indirect immunofluorescence. We conclude that differentiation of hESC can be induced by inhibition of cyclin dependet kinases but the underlying mechanism might not be associated exclusively with the elongation of the G1 phase of the cell cycle.