Translational repression of *E2F1* mRNA in carcinoma *in situ* and normal testis correlates with expression of the *miR-17-92* cluster

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Dear Editor,

The E2F1 transcription factor is a key regulatory factor of both apoptosis and cell cycle progression, and depending on cellular context, can act as either an oncogene or a tumour suppressor. Consequently, gene mutations that lead to deregulation of E2F1 activity are detected in a majority of human cancers.[1] Recently, an array study identified *E2F1* as upregulated in human testis containing preinvasive carcinoma *in situ* (CIS) cells when compared with normal human testis.[2] Apparently, supporting this, an E2F1 overexpressing mouse demonstrated generation of CIS-like cells in testis that were otherwise atrophying owing to massive E2F1-induced apoptosis of germ cells.[3]

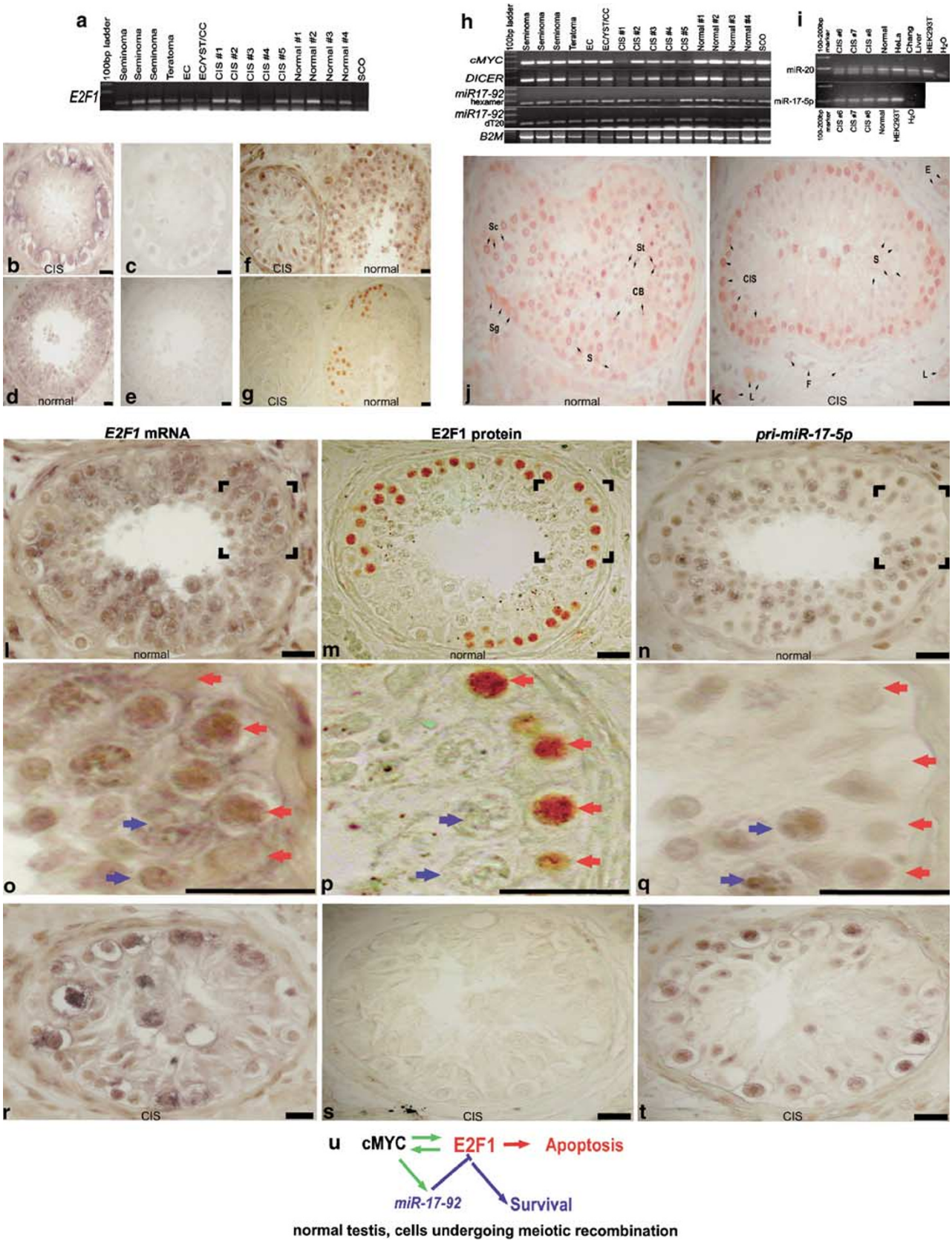
To further investigate and verify these data, we compared expression of *E2F1* on both the messenger and the protein level in human testis with and without CIS. Reverse transcription-polymerase chain reaction (RT-PCR) experiments confirmed the expression of *E2F1* mRNA in testis samples with and without CIS, as well as in various germ-cell-derived tumor types (Figure 1a). In contrast, there was little or no *E2F1* mRNA detected in testis tissue with Sertoli-cell-only suggesting that much, if not all, *E2F1* mRNA expression was derived from germ cells. Next, we used *in situ* hybridization (ISH) to identify the cell types expressing *E2F1*. The ISH experiments clearly showed expression of *E2F1* mRNA in CIS cells (Figure 1b and f, CIS), and in all germ cells in normal testis (Figure 1d and f, normal).

We then investigated expression of E2F1 protein in normal human testis and testis with CIS by immunohistochemistry (IHC). Surprisingly, E2F1 protein could not be detected in the investigated CIS samples, whereas in normal seminiferous tubules, numerous cells expressing E2F1 protein in the nuclei were readily detected (Figure 1g). However, when comparing expression of *E2F1* at the mRNA and protein level, it was immediately obvious that not all cells expressing *E2F1* mRNA also express E2F1 protein (Figure 1f and g). We identified the E2F1-positive cells as a subpopulation of spermatogonia (most likely B-spermatogonia) and early primary spermatocytes (preleptotene and leptotene). Later stages of germ cell maturation, including pachytene spermatocytes, secondary spermatocytes and spermatids, were negative. Apparently, *E2F1* expression is translationally repressed in specific cells in normal testis as well as in CIS cells.

Recently, translation of *E2F1* mRNA was discovered to be negatively regulated through the microRNA (miRNA) pathway by miRNAs from the *mir-17-92* cluster, which is expressed through transcriptional activity of cMYC[4]. Therefore, we analyzed the expression of components of this miRNA regulatory circuit (*cMYC*, *DICER*, *pri-hsa-miR-17-92*, mature miR-17-5p and miR-20a) in human testis. *cMYC* mRNA was detected in all samples by RT-PCR, as was the *miR-17-92* transcript (Figure 1h). Additionally, the *mir-17-92* reaction was run on two pools of cDNA prepared from either a dT_{20} oligo or a random hexamer as primer for first-strand synthesis. Both pools yielded equal results, indicating that the *miR-17-92* transcript is polyadenylated. The processed, functional forms of hsa-miR-17-5p and hsa-miR-20 were detected by RT-PCR run on size-fractionated (15–30 nt) RNA (Figure 1i).

The vital pre-miRNA processing gene *DICER* showed mRNA expressed in all investigated samples (Figure 1h), and DICER protein was detected in all germ and somatic cell types, albeit with varying levels of staining (Figure 1j and k). Highest levels of staining for DICER protein were found in CIS and germ cells, with almost equally high levels in Leydig and endothelial cells, whereas the lowest levels were observed in Sertoli cells. DICER was mainly present in the cytoplasm in normal germ cells and somatic cells and appeared to be localized to distinct foci in the cytoplasm of spermatids (Figure 1j), which probably correspond to the chromatoid body of male haploid germ cells described previously.[5] In contrast, the main expression in CIS cells appeared to be localized to the nucleus (Figure 1k).

As these results indicated that miRNA regulation of *E2F1* could indeed be active in human testis, we decided to follow the expression of *E2F1* mRNA and protein, together with *pri-miR-17-5p*, in individual cells using ISH and IHC techniques on consecutive sections of testis. Here, we saw *E2F1* mRNA expressed in all germ cells (Figure 1l), whereas only a subset of cells, near the edge of the tubule, expressed E2F1 protein (Figure 1m). Expression of *pri-miR-17-5p* was lowest in a subset of spermatogonia and early spermatocytes close to the edge of the tubule (Figure 1n), whereas *pri-mir-17-5p* expression increased with germ cell maturation proceeding toward the lumen, with strongest staining in pachytene spermatocytes (Figure 1n). This expression pattern is complementary to expression of E2F1 protein; that is, cells showing high expression of *pri-miR-17-5p* showed little, or no, expression of E2F1 protein, and vice versa. This pattern is seen more clearly when looking specifically at individual cells identifiable between the consecutive sections (Figure 1p and q). Cells showing strong E2F1 protein expression have low levels of *pri-mir-17-5p*, whereas cells containing little or no E2F1 protein have higher expression of *pri-mir-17-5p*.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Seminoma
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EC
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CIS #1
CIS #2
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r CIS
s CIS
t CIS
u cMYC
E2F1
Apoptosis
miR-17-92
Survival
normal testis, cells undergoing meiotic recombination

The situation in tubules containing CIS cells was easier to interpret as in most tubules CIS cells are the only germ cell type present. Although all CIS cells expressed *E2F1* mRNA (Figure 1r), no CIS cells contained detectable amounts of E2F1 protein (Figure 1s). In accordance, all CIS cells showed strong expression of *pri-miR-17-5p* transcript (Figure 1t), analogous to our results from normal germ cells.

Our results show evidence for miRNA-regulated inhibition of *E2F1* translation in a subset of cells (most notably pachytene spermatocytes) in normal testis, suggesting this may be a physiological mechanism operating during germ cell differentiation. It is noteworthy that E2F1's pocket protein partner, pRB, is expressed in the same subset of normal germ cells.[6] Pachytene spermatocytes represent a very distinct stage of germ cell maturation, where crossing-over of sister chromatids takes place to allow meiotic recombination. This process requires multiple double-strand DNA breaks, which in other cell types would trigger apoptosis if not instantly repaired. One of the known DNA repair mechanisms in pachytene spermatocytes is a massive activation of the ATM kinase, which targets numerous proteins, including E2F1.[7] We propose that the physiological inhibition of *E2F1* mRNA translation may be another important mechanism preventing apoptosis during meiotic recombination.

The pattern observed in CIS cells supports a role for miRNAs in cancer development. Recently, the investigation of miRNAs in testicular tumurs revealed that expression of miR-372 and miR-373 could lead to uncoupling of the p53-mediated checkpoint control, thus leading to increased proliferation and cancer development.[8] Our results indicate that the E2F1-regulated apoptosis pathway may also be impaired in CIS cells owing to the direct regulation of the *E2F1* mRNA by miRNAs from the *miR-17-92* cluster.

Additionally, results from other laboratories may indicate a reduced apoptosis in cancers, if they express the *miR-17-92* cluster.[9,10] In their work, it was undetermined what mechanism caused lowered apoptosis, but the work from O'Donnell *et al.*[4], together with our results, suggests that it may be the result of reduced E2F1 protein levels. Interestingly, miRNA inhibition of *E2F1* translation appears much more effective in the testis (essentially total loss of E2F1 protein) as compared with transfected Hela cells overexpressing the *miR-17-92* cluster (50% reduction of E2F1 protein).[4] We believe that biological differences, as well as the experimental setup, are the basis for this distinction. For example, miRNA-mediated translational regulation of *E2F1* may be more efficient on the *in vivo* mRNA levels of the testis as compared with Hela cells, which may have a more robust *E2F1* mRNA expression after generations of selective pressure for fast cell cycling. Alternatively, the transient nature and the varying efficiency of the transfection of Hela cells may be the cause of moderate reduction of E2F1 protein observed in the Hela cells.

Interestingly, mouse embryos show high expression of miRNAs from the *miR-17-92* cluster, with decreasing expression as they develop and differentiate.[11,12] CIS cells, which apparently originate during early fetal development from primordial germ cells or gonocytes that fail to fully differentiate,[13,14] retain many stem cell-like features and express markers such as OCT3/4,[15] KIT,[16] NANOG[17] and AP2 gamma.[18] Here, we show that CIS cells also express the *miR-17-92* cluster, thus supporting earlier speculation that expression of *miR-17-92* could be important for stem cell maintenance in the embryo.[9]

In conclusion, our results indicate that miRNA down-regulation of *E2F1* mRNA translation is part of the normal spermatogenesis, protecting meiotic cells from apoptosis as modeled in Figure 1u. In CIS cells, expression of the *miR-17-92* cluster may be a remnant of the stem-cell origin of CIS. Parallel with lung cancers[10] and B-cell lymphomas,[9] down-regulation of E2F1 protein expression in CIS cells may likewise propagate cancer development owing to the reduction of apoptosis.

Materials and Methods are available as Supplementary information.

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Figure 1 (**a**) RT-PCR detection of *E2F1* mRNA. EC: embryonal carcinoma. YST: yolk sac tumor. CC: choriocarcinoma. CIS: carcinoma *in situ*. CIS no. 1: from testis with 50–80% tubules harboring CIS cells, adjacent to EC. CIS no. 2: as no. 1, but adjacent to seminoma. CIS no. 3: 95% tubules harbor CIS cells, adjacent to seminoma/EC. CIS no. 4 and no. 5: 95% tubules harbor CIS cells, adjacent to normal tissue. Normal no. 1 and no. 2: normal testis. Normal no. 3: adjacent to EC/YST/CC mixed tumor. Normal no. 4: contained microliths. SCO: sertoli-cell only. (**b**) ISH detection of *E2F1* mRNA in tubules containing CIS cells. (**c**) Sense control to (**b**). (**d**) ISH detection of *E2F1* mRNA in normal tubules. (**e**) Sense control to (**d**). (**f**) ISH detection of *E2F1* mRNA and (**g**) protein in consecutive sections of human testis with (left tubule) or without (right tubule) CIS cells. Bars correspond to 20 μm. (**h**) RT-PCR detection of *cMYC* and *DICER* mRNA and *pri-miR-17-92* transcript in human tissues and tumors; lane description as in (**a**). (**i**) Detection of mature hsa-miR-17-5p and hsa-miR-20 in RNA from three different human CIS samples (CIS no. 6, 7 and 8), normal testis RNA and Hela, Chang Liver and HEK293 T cell lines. (**j**) DICER protein detection (IHC) in normal human tubules and (**k**) tubules with CIS. Sc: spermatocytes, Sg: spermatogonia, S: Sertoli cell, St: spermatids, CB: chromatoid body, CIS: carcinoma *in situ*, L: Leydig cell, F: fibroblast, E: endothelial cell. Bars correspond to 50 μm. (**l, m** and **n**) Consecutive sections of a normal human tubule. (**l**) ISH detection of *E2F1* mRNA; (**m**) IHC detection of E2F1 protein; (**n**) ISH detection of *pri-miR-17-5p*. Corner marks indicate areas magnified underneath. (**o, p** and **q**) Enlargements of (**l, m** and **n**). Red arrows indicate cells with expression of E2F1 protein, whereas the same cells have no or low expression of *pri-miR-17-5p*. Blue arrows indicate examples of cells with no expression of E2F1 protein and high expression of miR-17-5p. (**r, s** and **t**) Consecutive sections of a tubule containing CIS cells. (**r**) ISH detection of *E2F1* mRNA; (**s**) IHC analysis of E2F1 protein in CIS; (**t**) ISH detection of *pri-miR-17-5p*. Bars correspond to 20 μm. (**u**) Model of the function of *miR-17-92* expression in normal testis. cMYC and E2F1 are in a positive feedback loop, which if not regulated, would result in apoptosis owing to high levels of E2F1 protein. cMYC also activates expression of the *miR-17-92* cluster, which leads to the translational repression of *E2F1* mRNA. Regulated levels of E2F1 protein promote cell survival

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