Gene Silencing of Tead3 Abrogates Radiation-induced Adaptive Response in Cultured Mouse Limb Bud Cells

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Low dose/Radioadaptive response/Fetus/Mouse/TEAD.

There is a crucial need to better understand the effects of low-doses of ionizing radiation in fetal models. Radiation-induced adaptive response (AR) was described in mouse embryos pre-exposed in utero to low-doses of X-rays, which exhibited lower apoptotic levels in the limb bud. We previously described AR-specific gene modulations in the mouse embryo. In this study, we evaluated the role of three candidate genes in the apoptotic AR in a micromass culture of limb bud cells: Csf1, Cacna1a and Tead3. Gene silencing of these three genes abrogated AR. Knowing that TEAD3 protein levels are significantly higher in adapted cells and that YAP/TAZ/TEAD are involved in the control of cell proliferation and apoptosis, we suggest that modulation of Tead3 could play a role in the induction of AR in our model, seen as a reduction of radiation-induced apoptosis and a stimulation of proliferation and differentiation in limb bud cells.

INTRODUCTION

The effects of low doses of ionizing radiation (IR) on the embryo, such as adaptive response (AR), bystander effect, low-dose hyper-radiosensitivity and genomic instability, are of great concern, but knowledge of the molecular mechanisms involved in these phenomena still remains elusive.1,2) In particular, potential developmental and carcinogenic effects of low dose radiation are of crucial interest.3) Multiple in vitro and in vivo reports demonstrated the existence of an AR, whereby exposure to low doses or dose rate of IR reduced the biological effects of a subsequent challenging exposure.4) In an extensive series of studies, we described the existence of AR in mouse embryos exposed to X-rays in utero.5–11) AR was described as a reduction in prenatal deaths and congenital malformations in animals pre-exposed to low doses of X-rays6) and could be observed within two specific dose rate ranges of priming radiation.11)

There is still some speculation regarding the precise molecular mechanisms governing AR, because the existence and extent of AR depend on the endpoint, IR properties (dose, dose rate, linear energy transfer LET, time interval between priming and challenging doses) and experimental model. The involvement of DNA repair mechanisms,12,13) cell signaling pathways14) and reactive oxygen and nitrogen species (ROS/RNS)15–18) has been demonstrated. Recently, a lot of attention has been given to gene modulations resulting from exposure to IR,19) including at low-doses and low-dose rates; gene modulations were observed in mice exposed to long-term low-dose rate irradiation20) or in response to occupational exposure of radiation workers.21) It has been postulated that priming exposure activates genes involved in the protection against IR. Using microarrays, we recently analyzed transcriptome modulations in mouse embryos exposed to different low-doses of priming IR, which were or were not efficient in inducing AR in this model.5) Gene modulations resulting from AR-inducing and non-AR inducing priming exposure were compared and AR-specific gene regulations were identified. AR-specific genes were involved in various molecular functions and pathways (including DNA repair, cell signaling, developmental growth factors or tumor protein p53-related pathways).

Functional identification of the molecular pathways underlying AR in mouse embryos required the use of an experimental model, which would overcome the technical difficulties inherent to in utero studies. In order to investigate cellular and molecular properties of AR in fetal cells, we developed a model of micromass cultures of limb bud cells, which gave us the opportunity to reproduce in vitro biological properties of our in utero model. Indeed, it was
shown that the proliferation and differentiation of chondrocytes in micromass cultures followed closely what could be observed in vivo.\textsuperscript{22} It was shown that the teratogenic effects of IR in mouse embryos result from radiation-induced apoptosis; decreased apoptosis was observed in the limb buds of adapted embryos\textsuperscript{19} and in adapted cultured limb bud cells.\textsuperscript{13}

In this study, we evaluated the role of three AR-specific candidate genes (based on microarray data and on their biological role in apoptosis, cell proliferation and development) in the apoptotic AR of cultured limb bud cells: gene silencing of TEA domain family member 3 (Tead3), colony stimulating factor 1 (Csfl) and calcium channel, voltage-dependent, P/Q type, alpha 1A subunit (Cacna1a) by transient transfection with small interfering RNA (siRNAs) provided us some insights into the respective roles of these genes in AR.

\textbf{MATERIAL AND METHODS}

\textbf{Animals}

8 weeks-old ICR mice were purchased from SLC Japan Inc (Shizuoka, Japan) and maintained in a 12 h light/dark circle in the National Institute of Radiological Sciences (NIRS) animal facilities. After an acclimatization period of two weeks, females in estrus were mated with males overnight. The day on which a copulatory plug was found was counted as embryonic day 1 (E1).\textsuperscript{5} All experimental protocols involving mice were reviewed and approved by the Animal Care and Use Committee of the NIRS and were performed in strict accordance with NIRS Guidelines for the Care and Use of Laboratory Animals.

\textbf{Irradiation}

Irradiations were performed at room temperature with an X-ray machine (Pantak-320S, Shimadzu, Japan) operated at 200 kVp using a filter of 0.5 mm aluminum plus 0.5 mm copper. The dose rates were measured with an exposure rate meter (AE-1321M, Applied Engineering, Tokyo, Japan). Cells were exposed to a 0.3 Gy or 0.5 Gy priming irradiation (at a dose rate of 0.34 Gy/min) at E11 equivalent, and/or to a 4 Gy challenging irradiation (at a dose rate of 1.8 Gy/min) at E12 equivalent.

\textbf{Reagents and medium}

For cell culture, we used Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal calf serum, 100 IU/mL penicillin and 100 mg/mL streptomycin. CSF1, CACNA1A, TEAD3 and β-actin proteins were detected using the antibodies LS-C8599/8073109 (Lifespan Biosciences, Seattle, WA, U.S.A.), ab32642 (Abcam, Cambridge, MA, U.S.A.), LS-C30262/3017 (Lifespan Biosciences) and ab6276 (Abcam), respectively. horseradish peroxidase (HRP)-conjugated secondary antibodies 7071 and 7072 (for β-actin) and 7071 (for Csfl, CACNA1A and TEAD3) (Cell Signaling Technology, Danvers, MA, U.S.A.) were used for the chemiluminescent detection of the proteins.

\textbf{Selection of candidate genes}

Using DNA microarrays, we previously performed a global analysis of transcriptome regulations in adapted and non-adapted cells collected from whole mouse fetuses, after in utero exposure to priming irradiation.\textsuperscript{5} Gene expression in whole embryos after AR-inducing (0.3 Gy) and non-AR (NAR) inducing (0.5 Gy) priming exposure were compared. AR-specific gene modulations were identified and a functional analysis revealed the potential involvement of various molecular functions. In this study, we selected three candidate genes, which exhibited AR-specific modulation and were involved in cell signaling, apoptosis or cell proliferation: Csfl, Cacna1a and Tead3 (Table 1). Csfl is required for differentiating the monocyte-macrophage precursor cells into preosteoclasts and its activity is linked with phosphatidylinositol 3 Kinase (PI3K)/Akt signaling.\textsuperscript{23} It was also shown that Csfl promotes osteoclast survival through MEK/ERK pathway.\textsuperscript{24} Cacna1a encodes the subunit α of the Cav2.1 calcium channel, located principally in the central nervous system,\textsuperscript{25} and which plays a prominent role in regulating neurotransmitter release at central synapses and neuromuscular junctions.\textsuperscript{26} Other roles of Cav2.1 include cell survival\textsuperscript{27} and calcium signaling.\textsuperscript{28} The transcription factor Tead3 is expressed predominantly in the placenta and in embryonic tissues.\textsuperscript{29} The involvement of TEAD proteins

\begin{table}[h]
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\begin{tabular}{|l|l|c|c|c|c|c|}
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\textbf{Alias} & \textbf{Name} & \textbf{Gene modulations} & \textbf{Protein modulations} \\
& & \textbf{(whole embryo)} & & \textbf{(limb bud cells)} & \\
\hline
Tead3 & TEA domain family member 3 & 1.55 ± 0.18 & 1.07 ± 0.05 & 1.60 ± 0.09 & 0.86 ± 0.07 \\
\hline
Csfl & Colony stimulating factor 1 (macrophage) & 1.07 ± 0.15 & 1.97 ± 0.11 & 1.94 ± 0.08 & 3.31 ± 0.18 \\
\hline
Cacna1a & Ca channel, voltage-dependent, P/Q type, alpha-1A subunit & 0.68 ± 0.11 & 1.11 ± 0.02 & 0.68 ± 0.07 & 0.86 ± 0.07 \\
\hline
\end{tabular}
\caption{Gene and protein modulations after exposure to low-dose priming X-rays.}
\end{table}

Gene\textsuperscript{5} and protein modulations, compared to gene expression levels in non-irradiated whole embryos and limb bud cells, respectively, were measured. AR: AR-inducing priming exposure. NAR: non-AR inducing exposure.
in Hippo pathway has recently been characterized. In whole embryos, the growth factor Csf1 was up-regulated only in NAR conditions and the transcription factor Tead3 only in AR conditions; the calcium-channel Cacna1a was down-regulated only in AR conditions. Gene modulations of Csf1 were validated by RT-PCR.

**Micromass cultures and siRNA transfection**

Micromass cultures of fetal limb bud cells were established as described previously, with several modifications. Briefly, the whole buds of embryos at E10 were dissected and pooled in phosphate buffered saline PBS(−). Cells were dissociated by trypsinization and resuspended in DMEM. The cell suspension was then filtered through a 70-μm filter (Falcon 2350, Becton Dickinson, Franklin Lakes, NJ, U.S.A.). Cells were transfected with siRNA targeting Csf1, Cacna1a and Tead3 genes, and scrambled siRNAs which provided negative control (Table 2). Transient reverse transfection with lipofectamine was carried out in 24-well plates according to the manufacturer’s protocol (Invitrogen, Carlsbad, CA, U.S.A.). In brief, 6 pmol RNAi was diluted in 100 μL medium without serum in each well, then 1 μL of Lipofectamine RNAiMAX reagent was added. After 20 hour at room temperature, 5 × 10^5 limb bud cells in 500 μL medium without antibiotics were introduced in the wells and the calcium-channel Cacna1a was identified as apoptotic.

**Quantification of apoptotic cells**

In order to quantify apoptosis induction after irradiation, we followed the same protocol as described previously. Briefly, the whole buds of embryos at E10 were dissected and pooled in phosphate buffered saline PBS(−). Cells were dissociated by trypsinization and resuspended in DMEM. The cell suspension was then filtered through a 70-μm filter (Falcon 2350, Becton Dickinson, Franklin Lakes, NJ, U.S.A.).

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**Western blotting analysis**

Western blotting was performed according to the protocol provided by the manufacturers with minor modifications.

**RESULTS**

Radiation-induced apoptosis in limb bud cells

To study AR in limb bud cells, the time course of radiation-induced apoptosis in limb bud cells was measured during the 48 h following challenging irradiation (Fig. 1). The level of spontaneous apoptosis in non-irradiated cells was around 5%. Apoptosis peaked at 46% 6 h after exposure to a challenging dose of 4 Gy alone, then dropped to 18% 48 h later. A slight additive effect on apoptosis induction was observed in cells pre-exposed to 0.5 Gy at E11 equivalent, since apo-

### Table 2. siRNA sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>siRNA sequence (leading strand)</th>
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<tbody>
<tr>
<td>Tead3</td>
<td>5' AAAAAAAGGCCGUUUCUTT 3'</td>
</tr>
<tr>
<td>(scrambled)</td>
<td>5' GUCAGAUCAUAAGUUAAUTT 3'</td>
</tr>
<tr>
<td>Csf1</td>
<td>5' GACCCCGAGUACAGAGTT 3'</td>
</tr>
<tr>
<td>(scrambled)</td>
<td>5' GAGCGACGCGACGATCAATT 3'</td>
</tr>
<tr>
<td>Cacna1a</td>
<td>5' AGAUCACUGAAUGCCUCCTT 3'</td>
</tr>
<tr>
<td>(scrambled)</td>
<td>5' GGCCUCUCUAGAUAGUUTT 3'</td>
</tr>
</tbody>
</table>
ptosis levels in these cells were slightly above those in cells exposed to challenging IR alone. On the contrary, a protective effect on apoptosis was demonstrated in cells pre-exposed to 0.3 Gy: apoptosis levels were significantly lower in these cells than in cells irradiated with challenging dose alone or pre-exposed to 0.5 Gy. Therefore, we used the level of apoptosis 6 h after challenging IR as an endpoint for assessing AR in limb bud cells.

Expression of the candidate genes
We evaluated the expression of CSF1, CACNA1A and TEAD3 proteins in cultured limb bud cells at E12 equivalent by Western blot (Fig. 2). Modulation of CACNA1A and TEAD3 protein levels after priming and challenging irradiations were relatively similar to the modulation of gene expression in whole embryos exposed to priming dose: the amount of CACNA1A protein was lower for AR than for NAR conditions and it was the contrary for TEAD3. However, CSF1 protein levels were significantly higher than control for both AR and NAR conditions (although they were also significantly different for both conditions).

RNAi directed against Tead3, Csf1 and Cacna1 genes reduced their expression in cultured limb bud cells
siRNAs were transfected into limb bud cells at E10 equivalent, and their effects on altering protein levels were examined 24 h later by Western blot analysis. Repression efficiencies for Tead3, Csf1 and Cacna1a siRNAs were 95%, 69% and 77%, respectively, while protein levels were not modulated after transfection with scrambled siRNAs (Fig. 3). These results indicated that we successfully repressed the

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**Fig. 1.** Apoptosis induction after challenging irradiation in cultured limb bud cells. The percentages of apoptotic cells were measured according to time following challenging exposure. –■–: control cells. –○–: cells exposed to challenging 4 Gy irradiation at E12 equivalent only. ––: cells exposed to priming 0.3 Gy at E11 equivalent followed by challenging 4 Gy at E12 equivalent. –▲–: cells exposed to priming 0.5 Gy at E11 equivalent followed by challenging 4 Gy at E12 equivalent.

**Fig. 2.** Detection of TEAD3, CSF1 and CACNA1A proteins in fetal mouse limb bud cells exposed to priming (0.3, AR or 0.5 Gy, NAR) and challenging (4 Gy) radiation. Protein levels were visualized by Western blot (a). TEAD3 (b), CSF1 and CACNA1A (c) protein amounts were measured at E12 equivalent and compared to control (C). Cells were exposed to an AR-inducing priming dose of 0.3 Gy (AR), or to a non-AR inducing priming dose of 0.5 Gy (NAR) at E11 equivalent, then to challenging irradiation at E12 equivalent. The data represent the average of at least three experiments. Error bars represent standard error of the mean.
expression of the three candidate genes.

\textit{Influence of Csfl, Cacna1 and Tead3 repression on AR phenotype:}

In order to evaluate the effect of candidate genes repression on AR, we measured apoptosis in cultured limb bud cells 6 h after challenging irradiation (Fig. 4). As a negative control and to exclude possible effects from transfection, cells were also transfected with scrambled siRNAs for each candidate gene. Apoptosis levels in non-irradiated cells were not modified by transfection with siRNA. After exposure to challenging IR, apoptosis levels were not significantly different in transfected and non-transfected cells (47% of non transfected cells were apoptotic, while the percentages of apoptotic cells were 54%, 42% and 50% in cells transfected with siRNA targeting \textit{Tead3}, \textit{Csfl} and \textit{Cacna1a}, respectively).

As described above, cells primed with 0.3 Gy X-rays showed reduced apoptosis levels (33%), compared to cells exposed to challenging radiation alone. Transfection with siRNAs for \textit{Tead3}, \textit{Csfl} and \textit{Cacna1a} resulted in apoptosis levels which were not statistically different in cells exposed to challenging radiation alone and in cells exposed to both priming and challenging IR. In addition, after priming exposure, transfection with siRNAs for \textit{Tead3} and \textit{Cacna1a} resulted in apoptosis levels that were significantly higher than in non transfected cells. These results indicated that silencing of \textit{Tead3} and \textit{Cacna1a} effectively abrogated AR in limb bud cells.

\textbf{DISCUSSION}

We previously reported the existence of a radiation-induced AR in mice during late embryogenesis.\textsuperscript{3,6,8,11} In this study, we used siRNA-mediated gene silencing to assess the biological significance of AR-specific gene modulations. Three candidate genes were selected, based on their modulation ratios after priming IR (they demonstrated AR-specific modulations) and their biological role (they were involved in apoptosis, cell signaling, cell proliferation and/or development): \textit{Csfl}, \textit{Cacna1a} and \textit{Tead3}.

Micromass cultures of limb bud cells provide a valuable
experimental system in which chondrocytes proliferate and differentiate at the same rate as what can be observed in vivo.\textsuperscript{22) For practical reasons, siRNA assays were not performed in utero but in our system of cultured limb bud cells. Pre-exposure of these cells to 0.3 Gy X-rays (but not 0.5 Gy) resulted in apoptosis levels that were significantly lower than in cells exposed to challenging IR alone at 4 h to 8 h, indicating that AR in this model had similar characteristics to what we observed in utero. Therefore, measuring apoptosis at 6 h was a suitable endpoint for analyzing the effect of gene silencing on AR. Furthermore, because micromass cultures of limb bud cells are similar to the original in utero model, we can suggest that similar results might be observed in cultured limb bud cells and in the living embryo.

After exposure to priming dose, microarray analysis revealed that \textit{Tead3} was up-regulated only in AR-inducing conditions and \textit{Csf1} only in non-AR-inducing conditions (NAR); \textit{Cacna1a} was down-regulated in AR-inducing conditions. Thus these three genes expressed AR-specific modulations in the mouse embryo. When measuring the levels of the corresponding proteins in cultured limb bud cells after irradiation, TEAD3 was also up-regulated in adapted cells (but not in non adapted cells). However, CSF1 was up-regulated both in adapted and non-adapted cells (although CSF1 protein levels were significantly different for AR and NAR), while \textit{CACNA1A} protein levels were only slightly lower in adapted than in non-adapted cells. These results showed that modulations of \textit{CACNA1A} and TEAD3 protein levels in limb bud cells after exposure to both priming and challenging radiation might be similar to whole-body AR-specific modulations of the respective genes in response to low dose IR, suggesting that our previous microarray data were indeed useful in identifying molecular targets for AR in mouse embryos.

TEAD3 protein levels were significantly higher in adapted cells. Since apoptosis levels in cells transfected with siRNA for \textit{Tead3} and exposed to both priming and challenging IR were not lower than in cells exposed to challenging IR alone, we can conclude that gene silencing of \textit{Tead3} successfully repressed AR in this system. The molecular function of TEAD3 is not fully known yet, but TEAD proteins show a rather complex expression pattern during mouse development, suggesting a role for these proteins in developmental processes\textsuperscript{33), \textit{Tead3} gene expression was detected between E9.5 and E11.5 in the limb bud, as well as between E9.5 and E10.5 in the heart and pharyngeal arch.\textsuperscript{34) Recent investigations showed that TEAD family members act as mediators of the Hippo pathway, which plays a crucial role in the control of organ size.\textsuperscript{30) TEAD proteins mediate YAP/TAZ function (inhibited by upstream Hippo members) in promoting cell proliferation and migration as well as loss of contact inhibition through epithelial-mesenchymal transition (EMT).\textsuperscript{35} The increased TEAD3 protein levels in adapted cells are consistent with previous data showing increased proliferation and differentiation in adapted limb bud cells.\textsuperscript{11) Our results demonstrated that \textit{Tead3} gene silencing resulted in increased apoptosis levels in limb bud cells; similarly, YAP and TAZ loss of function in chick fibroblasts leads to increased apoptosis,\textsuperscript{36) suggesting a probable anti-apoptotic role of TEAD proteins as a part of the Hippo pathway. These findings are supported by \textit{Tead1/Tead2} double knockout mice embryos, which exhibit a phenotype similar to \textit{Yap} knockout embryos with decreased cell proliferation and increased apoptosis.\textsuperscript{37) Therefore we suggest that modulation of \textit{Tead3} could play a role in the induction of AR in our model, seen as a reduction of radiation-induced apoptosis and a stimulation of proliferation and differentiation in limb bud cells\textsuperscript{11) (Fig. 5). The role of YAP/TAZ/TEAD in organ

\begin{figure}[h]
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\includegraphics[width=\textwidth]{fig5.png}
\caption{The induction of radio-resistance by low-dose priming exposure in cultured fetal mouse limb bud cells. Exposure to priming 0.3 Gy X-rays (but not 0.5 Gy) results in the induction of AR, which represses apoptosis and stimulates proliferation and differentiation after exposure to 4 Gy X-rays challenging radiation. Induction of AR involves the expression of TEAD3, CSF1 and \textit{CACNA1A} proteins. TEAD3 might interact with YAP and TAZ to regulate downstream targets and induce AR.}
\end{figure}
size regulation is critical during development. However, it remains unknown whether proper and timely expression of specific genes is required; deregulation of genes such as Tead3 in adapted animals might result in developmental disturbances, as hinted previously. Indeed, adapted embryos presented high postnatal mortality and alterations in development and behavior. These results also raise the question of whether other long-term effects of AR, such as carcinogenesis. Emerging evidence support a link between YAP/TAZ/TEAD and cancer38); in human breast epithelial cells, TEAD proteins contribute to oncogenic transformation by mediating nuclear retention of TAZ.30,39) Furthermore, the identification of possible up-stream regulations of the Hippo pathway in response to IR will be of great interest. Such regulations are still unknown in mammals, but it was shown that IR activates the Hippo pathway and promotes apoptosis in D. melanogaster.40) No AR was observed when Csf1 gene expression was silenced. However, the data remained inconclusive because Csf1 protein was up-regulated in both adapted and non-adapted cells and because apoptosis levels after priming and challenging radiation were not significantly different between transfected and non-transfected cells. Furthermore, Csf1 gene silencing was not complete, since the repression efficiency for Csf1 siRNA was only 69%. A pro-survival function of Csf1 in osteoclasts and macrophages was recently described,41,42) but no such role of Csf1 was observed in cultured limb bud cells. Mice hosting an inactivating mutation in the Csf1 gene (csf1op)42) present reduced macrophages and osteoclasts numbers and show various abnormalities, suggesting that the regulation of Csf1 levels has potentially important consequences. Cacna1a gene silencing resulted in apoptosis levels similar to those in non-adapted cells (exposed to challenging IR alone), indicating that repression of Cacna1a also repressed AR; however, the precise involvement of CACNA1A protein in AR could not be drawn at the light of these results. The role of Cav2.1 in cell survival27,28) and calcium signaling28) suggests that the modulation of CACNA1A might influence radiation-induced apoptosis, but further study is necessary to clarify its role in various experimental models. It is known that mutations in the Cacna1a gene result in various neurological symptoms (ataxia, migraine), such as observed in the ataxic mouse rolling Nagoya.43) If a strong inhibition of Cacna1a was observed in the brain of adapted embryos, one can speculate that it might influence the subsequent development and behavior of adapted mice. Indeed, we demonstrated that surviving adapted fetuses exhibited impaired neonatal reflexes and altered adult behavior.44) Although our results concerning gene silencing of Csf1 and Cacna1a did not allow us to speculate about the molecular mechanisms directly linking the activity of CSF1 and CACNA1A proteins with AR, and even though these proteins were expressed at lower levels in adapted cells, we suggest that both CSF1 and CACNA1A proteins are required for induction of AR (Fig. 5).

CONCLUSION

AR-specific gene modulations were previously observed in mouse embryos exposed to priming radiation. Here we showed that silencing of Tead3 and Cacna1a genes repressed AR in a micromass culture of limb bud cells. We propose that up-regulation of Tead3 in adapted cells contribute to AR in mouse embryos, by repressing radiation-induced apoptosis. In view of the importance of YAP/TAZ/TEAD proteins in development and oncogenic transformation, future studies will provide additional insights into the role of these proteins and upstream signals in AR and low-dose response in the mouse embryo.

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