Preventing or reducing late side effects of radiation therapy: radiobiology meets molecular pathology

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Abstract | Radiation therapy has curative or palliative potential in roughly half of all incident solid tumours, and offers organ and function preservation in most cases. Unfortunately, early and late toxicity limits the deliverable intensity of radiotherapy, and might affect the long-term health-related quality of life of the patient. Recent progress in molecular pathology and normal-tissue radiobiology has improved the mechanistic understanding of late normal-tissue effects and shifted the focus from initial-damage induction to damage recognition and tissue remodelling. This stimulates research into new pharmacological strategies for preventing or reducing the side effects of radiation therapy.

Radiation therapy remains a cornerstone of modern cancer management, with an estimated half of all newly diagnosed cancer patients receiving radiotherapy at some point in the course of their disease[1]. Compared with surgery, radiation therapy has the advantage of being non-invasive and potentially organ preserving, although the functional outcome might be negatively affected by late side effects. Even in the era of molecular oncology, radiation therapy remains an attractive component of multi-modality therapy because it can be precisely modulated in time and space and provides effective tumour de-bulking in many cases.

All effective cancer therapies that have been developed so far are associated with a risk of various side effects and, as an increasing number of people are cancer survivors, preventing or reducing late side effects has increasingly become a priority. In the United States the National Cancer Institute and the Center for Disease Control estimate that 9.8 million people (3.5% of the population) were alive in 2001 after a diagnosis of non-skin cancer[2]. The number of cancer survivors in the United States more than tripled between 1971 and 2001, and this has further stimulated interest in the quality of life of this population. Relatively little is known about this issue, but it has been documented that the burden of late side effects on the physical and social functioning of the individual can be considerable[3–5].

The incidence and the severity or grade of a specific side effect depends on the details of how therapy is delivered but shows large variability among patients, even after strictly identical treatment. In the case of radiation therapy, the necessity to strike a balance between therapeutic benefit and associated toxicity became clear during the pioneering years of radiation therapy for cancer, and the systematic scientific study of the side effects of cancer therapy has been pioneered in this field[6]. Recently, the importance of this topic has been accentuated by the flurry of new radiation-treatment strategies in various stages of pre-clinical or clinical development. Experimental radiotherapies are not always better than the standard therapy — but they are often more toxic as they typically represent attempts to intensify therapy. Soares and colleagues[7] analysed data on 12,734 patients from 57 randomized controlled trials conducted between 1968 and 2002 by the Radiation Therapy Oncology Group. Overall, experimental and standard radiotherapies were equally successful (odds ratio for survival of 1.01; 99% CI 0.96–1.07; $P = 0.5$) whereas treatment-related mortality was worse in the experimental group of the trials (odds ratio 1.76; 99% CI 1.01–3.07; $P = 0.008$).

Whereas the typical side effects are systemic in the case of drug therapies, they are local or loco-regional after radiation therapy. In other words, the side effects become clinically manifest in tissues and organs that have been irradiated. Clinically, and to some extent biologically, it is important to make a distinction between early and late side effects. Early effects become manifest within a few weeks of the completion of a course of fractionated radiotherapy. These effects include skin erythema, dry or moist desquamation of the skin, mucositis, nausea and diarrhoea. Late effects are

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At a glance

- Around 50% of patients with solid malignant tumours receive radiation therapy with curative or palliative intent at some point in the course of their disease. Early and late side effects limit radiation dose and might affect the long-term health-related quality of life of the patient.
- The classical framework for discussing early and late side effects was the target-cell hypothesis: that the severity of side effects mainly reflected cell depletion as a result of the direct cell killing of a putative target cell leading to subsequent functional deficiency. This was the prevailing biological model until the mid 1990s.
- Recent research in radiobiology and molecular pathology has caused a change of paradigm, particularly in the understanding of late effects: radiation induces a concerted biological response at the cell and tissue level effected by the early activation of cytokine cascades.
- Fibrogenesis and excessive extracellular matrix and collagen deposition has a key role in the development and expression of many types of late effects. This can be seen as a wound-healing response gone wrong.
- Transforming growth factor-β is a key fibrogenic cytokine. Its activation, signalling pathway and downstream effects are understood in some detail and offer a number of potential targets for therapeutic intervention in the pathogenic process. This 'bottom-up' approach has benefited from the translation of findings from molecular pathology studies of other diseases characterized by the excessive development of fibrosis.
- Patient-to-patient variability in the response to radiotherapy represents a 'top-down' discovery strategy whereby clinical outcome data are linked with data from high-throughput assays.
- Radiogenomics is the study of genetic variation as an explanation for inter-individual differences in radiotherapy response. Most of the research so far has concentrated on single-nucleotide polymorphisms (SNPs) in selected candidate genes, but genome-wide approaches seem to be within reach in the near future.
- Advances in molecular radiation pathology combined with advances in clinical radiobiology, radiation therapy planning and delivery technology are likely to improve radiation therapy outcome within the next 5–10 years.

typically expressed after latent periods of months to years, and include radiation-induced fibrosis, atrophy, vascular damage, neural damage and a range of endocrine and growth-related effects. The pathophysiological and functional expression of this damage depends again on the tissue or organ affected. Radiation-induced second malignancies are of some concern, especially in patient populations with a long life expectancy, but will not be covered here. A recent review on this topic was published by Allan in *Nature Reviews Cancer*[8]. Although early effects are transient, that is, they settle after a few weeks[9], late effects tend to be irreversible or even progressive in severity[10].

Normal-tissue radiobiology has been through a veritable paradigm shift in recent years, mainly as a result of progress in molecular radiobiology that has led to an improved mechanistic understanding of the radiation pathogenesis of late side effects; this has opened intriguing new possibilities for the reduction or avoidance of such side effects.

From target cells to orchestrated response

To fully appreciate the significance of this paradigm shift and its possible therapeutic implications, it might be useful to briefly revisit the so-called target-cell hypothesis for normal-tissue effects of radiation that prevailed until the mid 1990s (BOX 1).

Under the target-cell hypothesis, the main effect of ionizing radiation on tissues and organs was thought to be a direct consequence of cell killing, resulting in the depopulation of crucial cell populations and subsequent functional deficiency. The pressure on this hypothesis came in part from cellular radiobiology studies that showed the importance of cell–cell communication in the processing of cellular radiation damage. After irradiating single cells with high-precision microbeams it was observed that cells in culture flasks in the vicinity of an irradiated cell could be killed without having been irradiated themselves, the so-called 'bystander effect'[11]. Further pressure came in the late 1980s and early 1990s when several research groups looked for a correlation between the *in vitro* radiosensitivity of normal human skin fibroblasts and late effects of radiotherapy, indirectly testing the clinical validity of the target-cell hypothesis. These studies were first conducted in patients with an atypically strong reaction to radiotherapy ('over-reactors')[12] and later in patients with reactions in the normal range. Early studies with a limited number of patients showed moderate support for a link between the radiosensitivity of normal human skin fibroblasts and late side effects[13–16], but when two larger confirmatory studies were reported, each comprising around 100 patients, there was no significant correlation between *in vitro* radiosensitivity and clinical response[17,18]. The explanation for this discrepancy is probably that many of the early studies were hypothesis generating, in other words the investigators deliberately varied the descriptors of radiosensitivity as well as the clinical endpoints, and reported the most significant findings from these exploratory analyses — a strategy that evidently leads to exaggerated claims of statistical significance.

Although the target-cell hypothesis remains a useful frame for discussing the early effects of radiotherapy[19], the inadequacy of this model became evident from the study of late side effects and, in particular, radiation-induced fibrosis — a clinically important side effect that never sat comfortably with the target-cell hypothesis. Clinically, radiation-induced fibrosis is characterized by reduced tissue flexibility, reduced compliance or strictures. Fibrosis is often associated with pain, neuropathy, reduced strength or restricted motion of joints and distal lymphoedema[20]. The corresponding histopathological picture typically shows that the normal tissue is partly atrophic, partly replaced by mesenchymal cells, and that there is excessive collagen deposition. It has long been clear that fibroblasts are the main cell type that deposit the extracellular matrix; but it was less obvious how the fibroblasts could also be the putative target cell for radiation-induced fibrosis. How could killing more fibroblasts lead to increased collagen production? Other important observations came from clinical studies that showed that non-cytotoxic drugs such as tamoxifen could increase the incidence of late radiation-induced fibrosis after radiotherapy[21–24]. All of this paved the way for a more active biological view of radiation effects, especially of late-responding normal tissues, and this is the topic of this Review.

Box 1 | **The heyday of the target-cell hypothesis**

Conceptually and empirically, the target-cell hypothesis was rooted in Puck and Marcus's successful method for growing single mammalian cells into colonies and their demonstration of a gradual loss of colony-forming capacity with an increasing single dose of ionizing radiation to the cells — published in a classical paper 50 years ago[148]. This became the basis for a flurry of *in vitro* studies of cellular radiobiology that led to the target-cell hypothesis, as formulated here in a textbook from 1987 (REF. 149): "the intensity of an effect [of radiation] usually reflects the proportion of cells irreversibly damaged by radiation as a result of lesions in their replicative mechanism." This paradigm has proven useful for understanding the early response of the haematopoietic system, the spermatogenic system[150,151] and epithelial tissues such as the mucosa that line the gastrointestinal tract or the skin[19].

Two mechanisms were thought to be behind the clinical expression of radiation damage, one being direct parenchymal cell loss and the other being the loss of vascular endothelial cells leading to infarcts and vascular collapse that subsequently affect organ function. The long latent period of late side effects, which in humans typically range from months to several years after the end of radiotherapy, was thought to be a 'silent' interval, during which the irreversible cellular damage was expressed in due course as cells underwent attempted mitosis resulting in mitotic death and subsequently compromised organ function.

The target-cell hypothesis left little room for interventions in radiation pathogenesis. Ionizing radiation was thought to create complex DNA lesions that were difficult to manipulate precisely, and the main route to improve the therapeutic ratio of radiotherapy in the early 1980s seemed to be to devise modified dose-fractionation schedules to exploit differences in the shape of the target-cell survival curve for tumours and late-responding normal tissues. Lowering the dose per fraction was seen as an important strategy for sparing late side effects relative to tumour effects. This led to a large number of randomized controlled trials of altered dose-fractionation, especially in squamous-cell carcinoma of the head and neck conducted in the 1990s[152,153]. These schedules were largely successful in creating a therapeutic differential between tumour and late normal-tissue effects, and in a way this body of clinical research represents the 'finest hour' of the target-cell hypothesis. Ironically, this happened at exactly the same time as the target-cell hypothesis came under substantial pressure from new biological and clinical observations that did not fit easily under the old paradigm.

It was discovered that the 'silent interval' between the irradiation and clinical expression of late normal-tissue injury is far from silent: soon after irradiation many cytokine cascades are activated, and these remain active throughout the phase of overt damage expression[25]. Although radiation-induced cell killing might have a role as a triggering event, it is now clear that there is an orchestrated, active biological response brought about by the early release of cytokines[26]. This response is mediated by various cell types, including inflammatory, stromal, endothelial and parenchymal cells actively responding through the release or activation of downstream cytokines, growth factors or chemokines.

Cytokine cascade
Cytokines, low-molecular-weight intercellular messenger proteins, are often produced in a cascade: one cytokine stimulates its target cell to secrete additional cytokines.

Chemokine
Small secreted cytokines that signal for various cell types to move in a specific direction, typically up the gradient of chemokine concentration.

Bleomycin
A chemotherapeutic antibiotic that functions by inducing DNA strand breaks, and which is therefore seen as a radiation-mimetic drug. Although the initial damage induction differs from that of radiation, it is probable that the mesenchymal-response pathway is similar for the two agents. It is often used to induce lung fibrosis in mouse models.

Molecular radiation biology of fibrosis

Among the late effects of radiation therapy, radiation-induced fibrosis is probably the most extensively studied. This is partly due to the importance of this reaction after clinical radiotherapy and partly because a lot has been learned from molecular pathology studies of wound healing and of human diseases characterized by the excessive formation of fibroses. Mechanistically, the early phases of fibrogenesis after irradiation can be seen as a wound-healing response characterized by an almost immediate upregulation of pro-inflammatory cytokines such as tumour-necrosis factor-α (TNFα), interleukins 1 and 6 (IL1 and IL6) and many growth factors in the irradiated tissue. Chemokines are released and these recruit inflammatory cells from the surrounding tissue into the irradiated volume. The interactions between the many proteins involved in the fibrogenic process are still not completely understood. However, useful insights into the *in vivo* function of the more than 100 proteins involved in wound healing have been gathered from genetically modified mouse models, gene knockouts or mice that transiently or permanently overexpress one of these proteins[27,28]. Normal wound healing (FIG. 1) is regulated by a complex balance between profibrotic proteins such as transforming growth factor-β (TGFβ)[29] and its downstream effector connective tissue growth factor (CTGF)[30] on the one hand, and antifibrotic proteins such as TNFα and interferon-γ (IFNγ) on the other[31]. The pro-inflammatory TNFα is expressed in macrophages during wound healing but downregulates the expression of matrix genes[32]. In addition, IFNγ is a pro-inflammatory cytokine released by T cells after a trauma, but has been shown to downregulate TGFβ and suppress collagen synthesis[33–35]: the intramuscular administration of IFNγ decreases bleomycin-induced lung fibrosis in a mouse model[35].

However, in contrast to normal wound healing, the radiation fibrogenic process is perpetuated over periods of many years (FIG. 1). Therefore, Martin *et al.*[36] have compared radiation fibrogenesis with a 'wound that does not heal' in analogy with other fibrotic diseases[37]. Understanding the homeostatic feedback control of normal wound healing — and why this is dysfunctional in radiation fibrogenesis — would clearly represent a breakthrough in the attempts to develop interventions.

***Transforming growth factor*-β.** TGFβ is a multi-functional cytokine, a 25 kDa polypeptide that is strongly profibrotic. When TGFβ was discovered it was classified as a growth factor, a term that reflects its involvement in the proliferation and differentiation of cells. At that time, the term cytokine was used to refer to signalling proteins in the haematopoietic and immune systems, but as cellular molecular biology evolved it was realized that the same kind of cell–cell signalling also has a fundamental role in

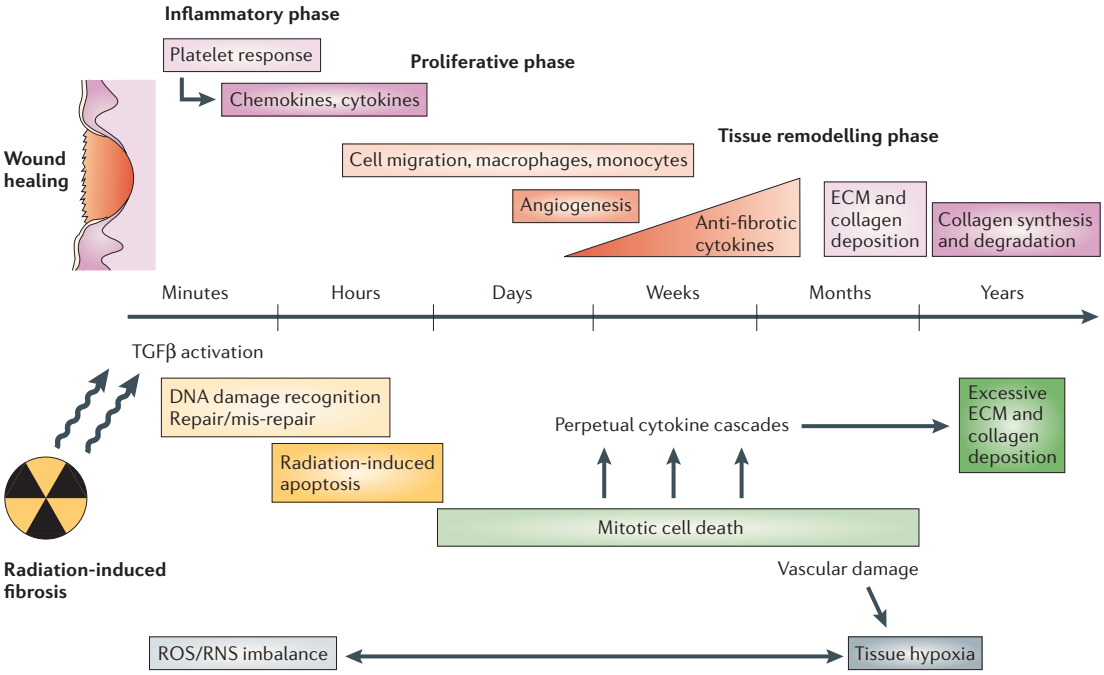

Figure 1 | **Phases of normal wound healing and radiation-induced fibrosis over time.** Normal wound healing (above the timeline) is a precisely orchestrated response to tissue injury, from the initial platelet response immediately after the trauma to the final remodelling of the scar tissue more than a year later. Radiation activates the whole wound-healing machinery (see also FIG. 2), but in addition to these processes the unique nature of radiation damage initiates a series of processes (below the timeline) that are distinct from those involved in normal wound healing. These processes span the whole timescale of normal wound healing, and it is probable that it is this continued interference with the normal control of wound healing that leads to the excessive deposition of extracellular matrix (ECM) and collagen that is characteristic of radiation fibrosis. ROS, reactive oxygen species; RNS, reactive nitrogen species; TGFβ, transforming growth factor-β.

solid tissues. TGFβ can usefully be classified as a cytokine and a growth factor. It has attracted much interest in fibrosis research, and there is a large and rapidly growing body of knowledge on this protein and its biological action. TGFβ belongs to a superfamily comprising over 60 proteins in multicellular organisms, with at least 29 of these encoded by the human genome and more than a dozen related molecules in invertebrates[38]. These proteins regulate a wide range of processes, including embryonic development, homeostasis, cell-cycle control and wound healing[31]. Dysfunction of the TGFβ system seems to be involved in various severe human diseases, including immunodeficiency, cancer, defective wound healing and a long list of fibrotic diseases in the kidney, liver and lung, as well as arteriosclerosis, rheumatoid arthritis and scleroderma. Interestingly, in the present context, TGFβ has a dual role in tumour suppression and tumour promotion[39,40]. TGFβ is a potent inhibitor of endothelial cell proliferation, in the mammary gland for example[41]. It has been proposed that during malignant progression breast cancer cells might become refractory to the growth-inhibitory effect of TGFβ, and that TGFβ, through its effect on the extracellular matrix, might promote invasion and metastasis in patients with advanced disease[41–43].

TGFβ exists in three isoforms (TGFβ1–3), and these show a high degree of homology between various species. Similar molecules are found in commonly used biological models such as *Caenorhabditis elegans* and *Drosophila melanogaster*[31]. TGFβ is secreted in latent form and is unable to bind to the receptor unless it is activated in the extracellular space by dissociation of the active mature TGFβ from the latency associated peptide (LAP)[44]. This means that a large extracellular pool of latent TGFβ can be rapidly mobilized after a triggering event. Ionizing radiation is one of the few exogenous factors that have been shown to induce TGFβ activation, and this happens within an hour or less of giving doses as low as 0.1 Gy[45,46]. The active TGFβ binds to pairs of two distinct transmembrane receptors (FIG. 2), TGFβR1 and TGFβR2, and it has been shown that TGFβR1 is unable to bind TGFβ in the absence of TGFβR2, and conversely, that the binding of TGFβ to TGFβR2 does not activate the signalling pathway in the absence of TGFβR1[47]. The biological advantage of this relatively complex — compared with other similar ligand–receptor systems — activation of the signalling pathway is not clear, but it probably contributes to the versatility of the transcriptional response to TGFβ. The signalling pathway itself is rather simple: the only established intracellular signalling effectors are the Smad proteins, of which there are five R-Smads (receptor-regulated Smads; SMAD1, 2, 3, 5 and 8) that are directly phosphorylated by the type I receptor, a co-Smad (SMAD4) that forms heteromeric complexes with the R-Smads and two inhibitory Smads (SMAD6 and 7) that antagonize TGFβ signalling[48]. The final transcriptional response

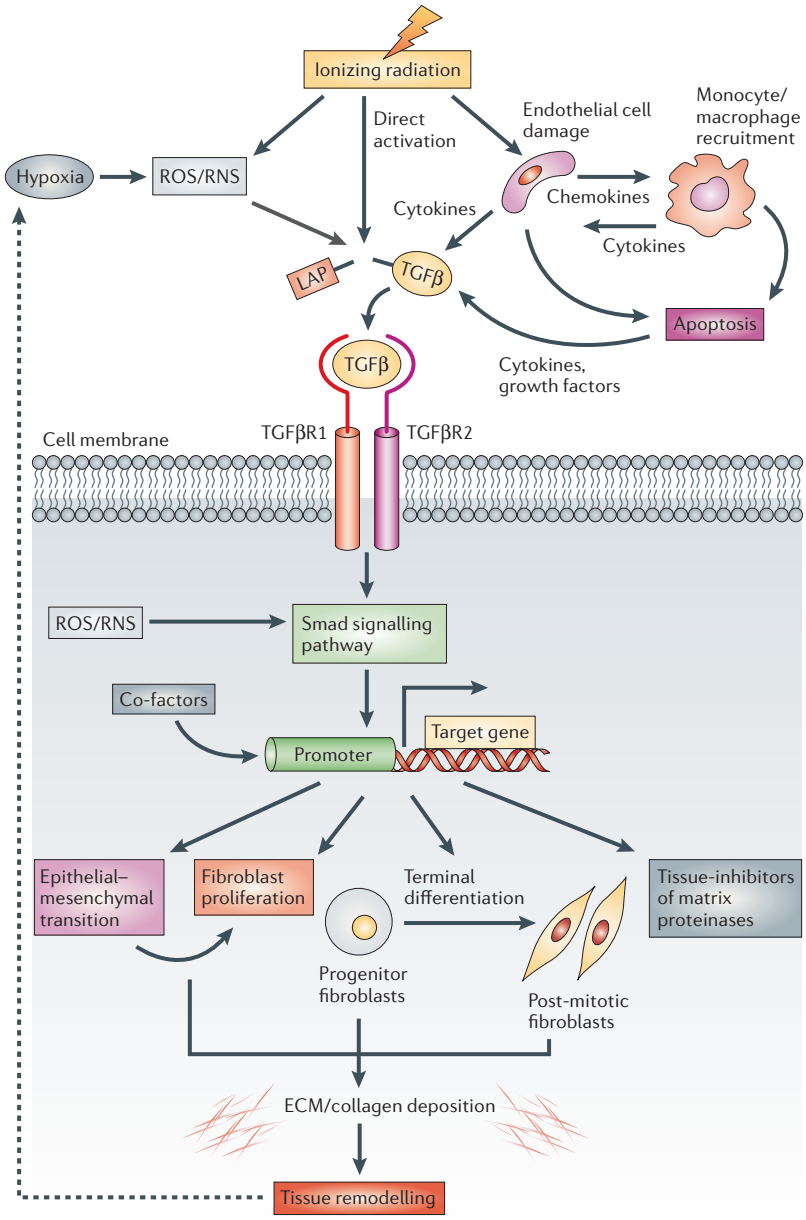

Figure 2 | **Key processes in radiation fibrogenesis.** Ionizing radiation directly activates transforming growth factor-β (TGFβ) through the dissociation of the latency-associated peptide (LAP) from the active mature form of TGFβ. Furthermore, radiation damages endothelial cells, which in turn initiate a cellular response that also leads to the release of pro-fibrotic cytokines, including TGFβ. A third main effect of radiation is that it perturbs the homeostatic control of the reactive oxygen and nitrogen species (ROS and RNS), which again leads to the activation of TGFβ and directly interferes with the Smad signalling pathway. These extracellular events activate the TGFβ signalling pathway, which in turn produces various transcriptional responses, all of which lead to increased extracellular matrix (ECM) and collagen deposition. The radiation-induced vascular damage and uncontrolled tissue remodelling can lead to tissue hypoxia, which could be one of the mechanisms perpetuating the fibrogenic response.

Tissue hypoxia
A pathological condition in which a tissue region is deprived of the normal physiological oxygen concentration.

of a specific target gene is determined by a number of DNA-binding transcription factors and various co-activators and co-repressors[38] (FIG. 2).

One of the many effects of TGFβ is that it promotes terminal differentiation along a lineage from proliferation-capable progenitor fibroblasts to postmitotic functional fibrocytes[49–52], and it has been suggested that the ratio between the number of colony-forming late and early progenitor fibroblasts would be correlated with the incidence of clinical fibrosis after radiotherapy. Two studies found some support for this hypothesis[53,54] but concluded that the association was too weak to be used as a potential predictive assay in the clinic.

But where do the fibroblasts come from in the first place? Epithelial–mesenchymal transition (EMT) of cells has long been recognized as an important part of embryonic development, but more recent data suggest that EMT occurs during wound healing and fibrogenesis in adult tissues[55]. In the case of renal fibrosis it has been estimated that more than a third of all disease-related fibroblasts stem from tubular epithelia[56]. It is probable that EMT has an important role in radiation fibrogenesis, and studies have shown that the loss of SMAD3 blocks EMT and reduces fibrogenesis[57]. There is also a strong experimental case for the mobilization of bone-marrow stem cells[58,59] and human mesenchymal stem cells[60] as an important element in the processing of radiation injury.

Although some of the signalling pathways are known in detail, it is still not clear why these cytokine cascades are continually perpetuated. One suggestion is that endothelial cell killing, which leads to vascular damage and subsequent tissue hypoxia, could drive radiation fibrogenesis[61,62]. This model is supported by experimental studies of fibrosis in the mouse lung showing that moderate hypoxia is present in the lung at 6 weeks after irradiation — long before pathological signs of restrictive lung injury become manifest[62]. There is also evidence in other fibrotic diseases that hypoxia is involved in their progression at least in the final stage of disease[63–65].

Reactive oxygen and nitrogen species. Another key element in fibrogenesis seems to be the homeostatic control of reactive oxygen and nitrogen species (ROS and RNS) in the cell. It has long been appreciated that ionizing radiation creates ROS in the cell, but after the discovery of the biological role of nitric oxide and its interaction with the superoxide radical $\bullet O_2^-$, oxidative and nitrosative stress are now seen as two sides of the same coin. It has been proposed that $\bullet O_2^-$ is the initiator and NO• and its derivatives the effectors of the activation of cytoplasmic signalling pathways after irradiation[66]. Until some 10 years ago it was a widely held view that the direct chemical action of ROS on DNA was an important mechanism behind radiation damage to the cell. However, it has been shown that the total concentration of ROS is dwarfed by the contribution from cell metabolism and other sources: a 1 Gy radiation dose produces less $\bullet O_2^-$ averaged over the cell volume than a human cell produces in 20 seconds[66,67]. Therefore, it would seem that it is the perturbation of the homeostatic control of ROS and RNS rather than the radical species themselves that drives the active biological response to oxidative and nitrosative stress. Superoxide dismutase (SOD) is the most important antioxidant enzyme, effectively catalysing the conversion of $\bullet O_2^-$ to H_2O_2 and thereby controlling the concentration of superoxide[66]. There is direct evidence that the SOD enzymes have

Reactive oxygen and nitrogen species
Highly reactive molecules that include oxygen or nitrogen, such as free radicals or other highly reactive forms (for example, singlet oxygen, a meta-stable state of oxygen with higher energy than the triplet ground state).

Telangiectasia
The visible dilation of small vessels under the skin or a mucosal surface that can occur after radiation therapy, perhaps as a result of radiation-induced cell killing and the loss of other small vessels in the area.

an important role in the molecular pathology of fibrosis[61,68–70]. The ROS and RNS pathway is an important interventional target in fibrotic disorders; see below.

The renin–angiotensin system. The renin–angiotensin system (RAS) regulates blood volume, arterial blood pressure and cardiovascular function. Although research into hypertension has traditionally focused on haemodynamic regulation by the RAS, more recent research points to the role of angiotensin II in vascular remodelling and fibrosis as a pathogenic factor in this disease[71,72]. The RAS has been known for some time to be implicated in kidney injury after irradiation, and studies in the early 1990s showed that the angiotensin-converting enzyme (ACE) inhibitor captopril reduced the level of kidney damage. Captopril contains a thiol group, and could therefore potentially function as a free-radical scavenger, but it has become clear that ACE inhibitors without a thiol group also work. Furthermore, it has been shown that these drugs are effective at doses that do not lower blood pressure and that other blood-pressure lowering drugs have no effect on radiation-induced kidney damage. Recently, Robbins and Diz[73] have reviewed the evidence that indicates that RAS might contribute to the development of radiation effects in other organs, for example the lung and the brain. As ACE inhibitors seem to decrease the risk of radiation effects irrespective of any systemic effects, it has been proposed that they act on locally generated angiotensin II, perhaps generated in response to oxidative stress. Angiotensin II has several functions, including an ability to upregulate TGFβ, and could therefore contribute to the fibrogenic response in irradiated tissue.

Pathogenesis of non-fibrotic late effects

Clinical and experimental animal data show that the fibrogenic pathway is heavily involved in the development and expression of many types of late side effects of radiation therapy, and it does seem to be a universal response to irradiation. Under the target-cell paradigm, the most important pathogenic mechanism was thought to be parenchymal cell depletion (BOX 1), and it is probable that this does have a role not only in early but also in late side effects of radiotherapy. However, in many cases this will be accompanied by a fibrotic–atrophic response, and it might not be possible to separate the relative contributions of the two in a given setting.

Interpatient variability in radiotherapy response

The preceding sections have described a mechanistic or 'bottom-up' approach, taking molecular pathology as the starting point and, hopefully, converging on clinically useful predictive factors and interventional targets. In view of the complexity of the biological response to radiation injury, a 'top-down' strategy, starting with individuals experiencing clinical side effects and attempting to discover a putative link with genetic or other predisposing factors, provides a complementary research avenue that has become increasingly appealing with the advent of powerful high-throughput assays.

Patients vary in their response to a specific course of radiotherapy[74–76], as shown by a number of clinical studies that have looked at the association between the grade of toxicity in separately irradiated volumes in a patient (BOX 2). This has fuelled research into phenotypic or genotypic predictive assays with the perspective of modifying therapy in radiosensitive individuals and perhaps intensifying therapy in relatively resistant cases.

As late effects are associated with both human suffering[4,5,77] and direct health-related costs[78], even costly treatment modifications would be justified in patients at high risk of developing late toxicity if they could be reliably identified by an assay. At the same time, the identification of predictive markers could point to new interventional targets for ameliorating late effects. Phenotypic assays have mainly concentrated on *in vitro* colony formation and assays of residual DNA damage after high radiation doses. Although some authors found support for an association between *in vitro* sensitivity and the severity of clinical normal-tissue effects[13–16], the observed correlations have been difficult to reproduce between studies[17,18], and the overall conclusion must be that these target-cell assays have limited or no use as a clinical screen for increased radiosensitivity[79]. This has turned much of the research interest toward genotypic assays. Also, the focus has broadened to include the early detection and repair of cellular damage and the tissue-remodelling response.

Box 2 | **Insights from studies of intra- and interpatient variability in radiation response**

Radiation effects are generally confined to the irradiated tissue volume, and this provides a unique opportunity to look at the correlation between the expressions of injury in two separately irradiated anatomical regions in the same individual. Several quite large clinical studies have been conducted with the aim of quantifying this correlation, and the main findings of these can be be summarized as follows. If the same clinical late effect, for example telangiectasia of the skin or subcutaneous fibrosis, is scored in two different radiation fields, there is a significant correlation between the level of over- or under-expression (relative to the average reaction in patients receiving the same treatment) of the reaction in the two fields[76,154,155]. The intra-patient correlation between different clinical endpoints, such as early skin erythema on the one hand and telangiectasia or fibrosis on the other[156], or between two late endpoints, telangiectasia and fibrosis[154], is much lower and is not significantly different from zero. Taken together, these studies indicate that there is some as yet unknown genetic or physiological patient-related factor that alters the expression of a specific type of normal-tissue reaction after radiotherapy, but that this factor might not be uniformly expressed in various cells or tissues. The magnitude of the intra-patient correlation for skin telangiectasia is considerable: in one study it was estimated that the deterministic variation in the clinical expression of this endpoint accounted for 81% of the total variability in response[76]. This clearly encourages further studies that aim to define these factors and use them either as predictive factors or as potential targets for intervening in radiation pathogenesis.

The hypothesis that genetic variations have a role in determining radiosensitivity has found support in the observed hyper-radiosensitivity associated with some rare genetic syndromes[80] such as Nijmegen breakage syndrome, Fanconi anaemia and ataxia telangiectasia (AT). AT is prototypical of these diseases, a rare autosomal recessive disorder characterized by progressive neuronal degeneration, immunological deficiency and an increased incidence of cancer[81,82]. The gene mutated in AT (*ATM*) was identified in 1995 (REFS 83–85), and encodes a kinase that amplifies the DNA-damage signal induced by DNA double-strand breaks[86]. ATM and its downstream kinase CHK2 phosphorylate several targets that regulate DNA repair, cell-cycle checkpoints and apoptosis. The *in vitro* radiosensitivity of skin fibroblasts from AT homozygous patients is typically threefold higher than that of normal human fibroblasts[87], and clinical case studies show that these patients have an extreme normal-tissue reaction to radiotherapy and might achieve local tumour control at a fraction of the radiation dose used normally[88–90]. The incidence of AT has been estimated at <1 per 40,000 live births, which means that about 0.5% of the population is heterozygous for a germline mutation in *ATM*. The *in vitro* radiosensitivity of fibroblasts from *ATM* heterozygous patients tends to be somewhere between that of *ATM* homozygous patients and that of normal human skin fibroblasts. *Atm* heterozygous mice have increased sensitivity to radiation-induced cataracts[91]. Because of the link to breast cancer susceptibility, it has been suggested that *ATM* heterozygous patients might constitute up to 8% of all patients with breast cancer[82]. However, a recent study showed that among patients with early-onset breast cancer (defined as onset before 45 years of age) the prevalence of protein-truncating germline *ATM* mutations was just 8.5% (REF. 92). Therefore, it seems unlikely that these cases will represent a significant proportion of cases with severe late effects of radiotherapy. Indeed, protein-truncating *ATM* mutations do not seem to be more prevalent among patients with breast cancer who have a pronounced reaction to radiotherapy than among patients who have a normal reaction[93–95]. This leaves the possibility that minor genetic variations could influence radiation response, and this is discussed below.

Among the downstream targets of ATM and CHK2 is the tumour-suppressor gene *BRCA1* (REFS 96,97). Germline mutations in *BRCA1* have been estimated to account for around 5% of sporadic breast cancer cases[98,99], and women with such a mutation have a 46% cumulative risk (95% confidence limits 39% and 54%)[100] of having developed breast cancer before the age of 70. Again, it has been speculated that these patients might constitute a radiation-sensitive subset of patients with breast cancer in the clinic. However, two small studies[101,102] and a large case–control study, which matched 71 women with a *BRCA1* and/or *BRCA2* mutation and stage I or II breast cancer with 213 women with sporadic breast cancer[103], did not find any increased risk of early or late radiotherapy toxicity in the cases with mutated BRCA.

Radiogenomics: design of studies

Radiogenomics is the study of genetic differences in the response to radiation[104], coined in analogy to pharmacogenomics, the study of patient-to-patient variability in the effectiveness and toxicity of drugs. This is an emerging field of research in which attempts at investigating a possible genetic background for variations in clinical radio-responsiveness have concentrated on single nucleotide polymorphisms (SNPs) in selected candidate genes and the screening of multiple genes using gene-expression arrays.

SNPs have attracted considerable interest in cancer susceptibility studies[105,106] and pharmacogenomics[107], and there are now a dozen or so studies on SNPs in radiogenomics (reviewed in REF. 108).

There are two broad classes of approaches to mapping genes associated with a specific phenotype: genome-wide and candidate-gene studies[109–111]. In the case of late side effects after radiotherapy, it can be argued that low odds ratios are probably not clinically relevant because dosimetric uncertainties will tend to dominate over a biological effect conveying a low relative risk. If a treatment that delivers 70 Gy in 2 Gy fractions to a patient is associated with a 10% risk of radiation-induced fibrosis, then a carrier of an SNP with an odds ratio of 2 would experience an 18% risk of this complication. But using clinical estimates of the steepness of the radiation dose–response curve[112], this is equivalent to the risk experienced by a non-carrier who receives a dose of 73.5 Gy, that is, a 5% higher absorbed dose — a dose difference that is not too far from the achievable precision in radiation dose delivery in clinical practice. If we concentrate the search on SNPs with odds ratios of 2 or more, searching for side-effect susceptibility SNPs with a minor allele frequency of 3%, for example, would require a sample size of some 1200–1500 individuals[111]. Although this is an order of magnitude larger than currently published candidate-gene studies, it is not unrealistic to conduct such a study, as is discussed further below.

The alternative design, candidate-gene studies, is a hypothesis-driven approach that specifically tests the relevance of SNPs in genes that are already known to be involved in radiation-related damage induction and processing or in tissue remodelling. Furthermore, it seems logical to concentrate efforts on SNPs that give rise to a non-conservative amino-acid change in the final gene product or are located in regulatory regions that possibly affect gene expression or protein-secretion rate[113]. A recent review by Andreassen[108] included eight studies of the possible association between SNPs and clinical normal-tissue response to radiotherapy. Although some of these studies did obtain consistent results when looking at the same SNP, the overall impression is that there is so much variability in clinical endpoints, in study design and in the exact hypothesis tested, that a synthesis of all these data is difficult. Many of the original reports include a substantial element of exploratory data analysis. As an example, the largest study conducted to date, including 446 individuals, looked at six SNPs in three repair genes and found that none of them were directly associated with the risk of developing early side effects of radiotherapy[114].

Nijmegen breakage syndrome
A rare heritable disease characterized by an abnormally small head and underdeveloped brain, associated with chromosomal instability and a predisposition to cancer, especially lymphomas.

Fanconi anaemia
A rare heritable disease in which the bone marrow fails to produce platelets, red or white blood cells or a combination of the three. It is associated with a predisposition to cancer, particularly leukaemia.

Ataxia telangiectasia
A rare heritable disease characterized by progressive dysfunction of the cerebellum, the part of the brain that coordinates voluntary motion, and a predisposition to cancer, particularly lymphomas and leukaemia.

Single nucleotide polymorphisms
(SNP) An inter-individual variation in the DNA sequence that involves the substitution of a single nucleotide that occurs in more than 1% of the population.

Candidate gene
A gene whose function indicates that it could be mechanistically involved in a specific process, such as radiation-damage repair or tissue remodelling.

Genome-wide SNP genotyping
A strategy for trying to discover associations between SNPs in any human gene and a specific phenotype; for example, patients showing atypically strong side effects after radiotherapy.

However, looking at the joint effects of carrying more than one SNP, a pair of SNPs was identified that was significantly associated with early side effects in normal-weight but not in overweight patients. Clearly, this is a data-generated hypothesis — and potentially a spurious finding — that needs validation in an independent study before its significance can be established.

One obvious methodological issue that plagues virtually all the studies published so far is multiple comparisons. For example, in a series of 41 patients that received postoperative radiotherapy for breast cancer, Andreassen *et al.*[113] assessed 17 specific SNPs in *TGFβ1*, *SOD2*, *XRCC1*, *XRCC3* and *APEX*, and found that 7 of these were associated with a significantly increased risk of developing severe subcutaneous fibrosis. However, applying a Bonferroni correction to the published data in their paper shows that only one of these (XRCC3 codon 241 Thr/Met) remains significant after the correction. Therefore, these early studies should be seen as hypothesis-generating, and subsequent confirmatory studies are much needed. In his review[108], Andreassen refers to an unpublished confirmatory study including 120 patients from the same institution as their initial series of 41 cases, but apparently none of the previously identified SNPs were significantly associated with an increased risk of subcutaneous fibrosis in the second study. The combination of hypothesis-generating and hypothesis-testing studies is clearly the way to go. The early experience in this research field is sufficiently positive to warrant further studies — and further studies are much needed to arrive at true-positive SNPs associated with radiotherapy side effects.

The transcriptional response of normal cells and tissues to radiation has been the subject of a few studies using cDNA microarrays[115–117]. Although these are also powerful discovery tools in radiation research[118] there is still a long way to go before we have a gene signature of value in clinical response prediction.

A final caveat should be mentioned here: as a large part of the variability in response to radiation therapy is explained by dosimetric and patient-related factors (BOX 3), a prerequisite for successful studies in radiogenomics is a high level of quality control of radiation therapy planning and delivery and a careful recording of patient-related factors that might affect clinical outcome[119,120].

Interventions to prevent late radiotherapy effects

Under the target-cell hypothesis, the most important means of creating a differential between tumour and late normal-tissue effects was the modulation of the dose-fractionation schedule. Pharmacological interventions concentrated on cytoprotective compounds such as the free-radical scavenger amifostine[121]. Clearly, for this kind of drug to convey a therapeutic advantage it would require some selectivity for the protection of normal-tissue cells relative to tumour cells. The US Food and Drug Administration (FDA) has approved amifostine in the following, quite specific, indication (NDA 20-221/S-020): "to reduce the incidence of moderate to severe xerostomia in patients undergoing post-operative radiation treatment for head and neck cancer, where the radiation port includes a substantial portion of the parotid glands." Still, although amifostine is under continued investigation in a number of clinical indications, the role of this drug remains controversial: in part, because amifostine is associated with fairly marked side effects of its own[122] — hypotension, vomiting and allergic reactions — and in part because a substantial body of preclinical data suggest that amifostine induces some degree of tumour protection in addition to normal-tissue protection[123]. Reduced tumour response has not been seen in the clinical trials, but it can be argued that these have not had sufficient statistical power to resolve a clinically relevant tumour-protection effect. All of this has led to the FDA recommending against the use of amifostine in patients receiving primary radiotherapy outside controlled clinical trials. This lack of proven specificity is the main issue surrounding the use of cytoprotective agents.

As our understanding of the pathogenesis of late radiation effects improves, specific interventional strategies are being developed that target all the contributing pathogenic pathways for late effects[61,124,125]. Early intervention during the initial phase of the cytokine cascade might be highly effective in modulating the subsequent cell and tissue response. Perhaps the most intriguing target is the TGFβ pathway because of its key role in radiation fibrogenesis and its proposed dual role as a suppressor and promoter of malignant progression[41,43,126]. This makes TGFβ a high-risk target for intervention on the one hand, but a target with anticancer potential on the other — patient selection could turn out to be a key factor. Several TGFβ-targeting strategies are in clinical trials as cancer therapies[39,126,127], and these include TGFβ antisense oligonucleotides, TGFβ antibodies and small-molecule inhibitors of the signalling pathway. Other strategies currently in pre-clinical development involve the silencing of the TGFβR2 receptor using a dominant-negative receptor, or inhibiting TGFβ signalling using the extracellular soluble domain of the TGFβR2 receptor.

Proof of principle for the prevention or amelioration of radiation fibrogenesis through TGFβ targeting has been obtained from pre-clinical studies. The classical study by Giri and colleagues[128] was published more than 10 years ago, and showed that the administration of an antibody against TGFβ reduced the severity of bleomycin-induced lung fibrosis. Other studies have established that the delivery of soluble TGFβR2 receptor by gene therapy with an adenoviral vector[129] or by intra-tracheal instillation[130] reduces the risk of bleomycin- or radiation-induced lung injury in rodent models. Also, the signalling pathway constitutes a potential target: *SMAD3* knockout mice show resistance to radiation-induced fibrosis[131], and the Smads constitute potential targets for drug therapies[127]. Finally, there are several new small-molecule inhibitors in various stages of development (for example, SM305 (REF. 132) and halofuginone[133]) that look promising in pre-clinical models. Several of these strategies are in clinical trials as potential interventions in fibrotic disease or as prophylaxis against excessive scarring after surgery[134]. No doubt some of these will enter clinical trials in radiotherapy patients within the next few years.

Bonferroni correction
A multiple-comparisons correction that is applied to reduce the chance of spurious ('false-positive') findings when several statistical tests are conducted to analyse a data set.

Xerostomia
Dryness of mouth caused by reduction in the secretion of saliva, a possible side effect of radiation therapy for cander of the head and neck region.

Box 3 | **Predisposing factors for radiotherapy-related side effects**

There is some support for an effect of several patient and life-style related factors on the expression of normal-tissue damage after radiotherapy[75,157]. These are of interest partly because they provide some pathogenic insight and partly because they are confounding factors in studies of clinical radiation effects. Unfortunately, much of the published literature consists of case reports or poorly designed retrospective studies[158]. One of the most consistent findings is that an increase in a patient's age is associated with an increase in the risk of developing reduced organ function after radiotherapy[20,159,160], but does not affect endpoints[9,161] that do not directly depend on the physiological reserve capacity before irradiation. Further support for the hypothesis that reserve capacity is important for the ultimate risk of functional problems comes from studies that show a direct, inverse relationship between pre-radiotherapy function and the risk of functional problems after therapy[159,160]. Of special mechanistic interest are connective tissue diseases, a group of diseases characterized by common features like the inflammation of skin, joints or other organs, and altered immunoregulation resulting in auto-antibodies and the dysfunction of cell-mediated immunity. These diseases include rheumatoid arthritis, systemic lupus erythematosus, dermatomyositis, polymyositis and systemic sclerosis. A recent systematic review found significant support for an increased incidence of late side effects of radiotherapy in patients with connective tissue diseases[158]. However, the association was relatively weak, and most studies in the literature are plagued by issues with the methods used. Further progress in this field will require large, prospective studies with high-quality dosimetry and specific follow-up procedures for scoring normal-tissue effects.

Another interesting target is the ROS and RNS pathway discussed above. Both Mn SOD delivered by an adenovirus vector[135] and inducible NO synthase inhibition[136,137] reduce fibrogenesis. Of particular interest from a biological and clinical point of view are a number of clinical studies that suggest that manifest radiation-induced fibrosis can be reversed using antioxidant therapies[61]. Initially, liposomal Cu and Zn SOD was tried with some clinical benefit[138,139], but more recent studies have shown significant regression of clinically marked fibrosis using pentoxifylline and vitamin E[140,141]. Treatment was continued for several months or even years, depending on the response of the patient, and was well tolerated. Although most patients obtained at least some regression of their fibrosis, few obtained a complete remission, and there was evidence of a rebound effect after the end of treatment[140]. Further optimization of this therapy is required, but the demonstration of significant regression of fibrosis at long intervals after initial onset is of significant mechanistic and clinical interest.

Collagen deposition is a result of an imbalance between extracellular matrix synthesis and degradation, and the degradation step might be as interesting as the synthesis step as a potential interventional target in fibrogenesis[142].

Perhaps it is appropriate to conclude this discussion of the molecular targeting of radiation-induced late effects by observing that although much of the emphasis over the past 5–10 years has been placed on tissue remodelling as a crucial element in radiation pathogenesis, the damage-induction step — that is the initial cellular processing of damage — remains relevant as a target for intervention. Interventions that aim to reduce the initial cell killing would also reduce the cellular and tissue-damage response. Except for free-radical scavengers, discussed above, none of these strategies are in clinical trials as a means of preventing the side effects of radiotherapy.

Finally, although a thorough discussion of this exciting field is beyond the scope of the present paper, the potential of stem cells in regenerative medicine is of huge interest in the amelioration of late effects of radiotherapy[143,144], and this provides yet another interventional target for the future.

The way forward

Molecular pathology is a powerful partner for traditional clinical and experimental radiobiology, and this has opened new research avenues that are being actively pursued: mechanistic studies that aim to identify drugable targets in the pathogenic pathway, and high-throughput assays applied to clinical samples as target-discovery tools or that aim to develop a clinically useful late-effects signature. A third active research field, not covered in any detail in this Review, is clinical normal-tissue radiobiology, which documents and records late effects after radiotherapy alone or in combination with other modalities and takes new interventional strategies into prospective clinical trials.

There is an intimate relationship between radiation-dose fractionation, spatial dose distribution and the clinical outcome of radiation therapy. Effective normal-tissue response modifiers or reliable predictive assays of normal-tissue effects would enable the intensification of tumour dose and/or the application of dose distributions with a higher probability of achieving loco-regional tumour control. This second perspective is becoming particularly exciting owing to the improved technologies for radiation therapy planning and delivery[145,146].

With dramatically improved research opportunities springing from progress in molecular biology, one limiting factor in many current studies in this field is the quality of the clinical data analysed. Although composite patient-related endpoints are of obvious clinical relevance, more specific normal-tissue endpoints are likely to be more informative in mechanistic studies[6]. New radiation therapy technology and new treatment philosophies create non-uniform normal-tissue dose distributions that vary widely from patient to patient, and this will have a significant impact on side effects, irrespective of biological factors. Quality assurance and precise 3D dosimetry is required to adjust for the confounding effect of dose distribution[78].

Finally, large sample sizes will be required, especially in studies of high-throughput assays. With the current level of ambition for statistical power, databases and normal-tissue banks that are comprised of several thousand individuals are being established[120]. The most comprehensive of these at the time of writing is the European GENEPI normal-tissue bank[147], which is currently comprised of more than 5,000 cases.

The huge research efforts are starting to pay off, and it seems safe to predict that progress in normal-tissue radiobiology and molecular pathology will lead to an improvement in the therapeutic efficacy of radiation therapy and improve the quality of life of long-term cancer survivors in the next 5–10 years.

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Competing interests statement
The author declares no competing financial interests.

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