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RAGE signaling deficiency in rhabdomyosarcoma cells causes upregulation of PAX7 and uncontrolled proliferation

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ABSTRACT

Embryonal rhabdomyosarcomas (ERMSs) show elevated levels of PAX7, a transcription factor that marks quiescent adult muscle stem (satellite) cells and is important for proliferation and survival of activated satellite cells and whose timely repression is required for myogenic differentiation. However, the mechanism of PAX7 accumulation in ERMSs and whether high PAX7 causes uncontrolled proliferation in ERMS remains to be elucidated. The receptor for advanced glycation end-products (RAGE, encoded by AGER) transduces a myogenic and anti-proliferative signal in myoblasts, and stable transfection of the ERMS cell line TE671, which does not express RAGE, with AGER results in reduced proliferation and formation of tumor masses in vivo, and enhanced apoptosis and myogenic differentiation. Herein, we show that RAGE expression is low or absent in human ERMSs. We also show that in ERMS cells (1) PAX7 accumulates owing to absent or low RAGE signaling; (2) elevated PAX7 levels reduce RAGE expression and levels of MyoD and myogenin, muscle-specific transcription factors required for myoblast proliferation arrest and differentiation, respectively; (3) PAX7 supports myoblast proliferation by reducing the levels of MyoD, primarily by promoting its degradation; and (4), when ectopically expressed in ERMS cells, that RAGE upregulates myogenin which upregulates MyoD and downregulates PAX7, with consequent inhibition of proliferation and stimulation of differentiation. Thus, failure to express RAGE and, hence, MyoD and myogenin above a critical level in ERMS cells might result in deregulated PAX7 expression leading to uncontrolled proliferation and, potentially, to rhabdomyosarcomagenesis.

KEY WORDS: Embryonal rhabdomyosarcoma, PAX7, Proliferation, MyoD, Myogenin, RAGE

INTRODUCTION

Embryonal myogenesis and skeletal muscle regeneration are driven by the timely activation in myoblasts of the muscle-specific transcription factors Myf5, MyoD, myogenin and MRF4, of which myogenin is essential (Brack et al., 2007; Buckingham, 2006; Chargé and Rudnicki, 2004; Kuang et al., 2006; Yin et al., 2013). These factors operate downstream of paired-box (Pax) transcription factor 7 (PAX7) that is involved in the survival and proliferation of myoblasts, during development, and of satellite cells (SCs), the adult stem cells of skeletal muscles, during muscle regeneration. In normal conditions, PAX7 levels in myoblasts are tightly controlled: PAX7 drives the induction of MyoD in activated and proliferating SCs, with MyoD in turn driving myogenin expression in differentiating myoblasts (Sabourin et al., 1999; Seale et al., 2000; Zammit et al., 2006); PAX7 becomes repressed at the onset of adult myogenesis; and, PAX7 persistence in myoblasts results in prolonged proliferation and defective muscle regeneration (Zammit et al., 2004). However, absence of PAX7 compromises SC expansion following acute muscle injury (Kuang et al., 2006) and, in striking contrast, PAX7 overexpression results in myoblast proliferation arrest (Olguin and Olwin, 2004), although this latter conclusion has been challenged (Zammit et al., 2006). Thus, mechanisms must exist that timely and precisely regulate PAX7 levels in normal conditions.

Rhabdomyosarcoma (RMS), an aggressive malignant pediatric soft-tissue sarcoma, is thought to arise from cells of the skeletal muscle lineage (Breitfeld and Meyer, 2005; Rubin et al., 2011; Saab et al., 2011; Tiffin et al., 2003). RMS is divided into two major histological subtypes, embryonal and alveolar (Hettner and Wagers, 2010); embryonal RMS (ERMS) shows the phenotypic and biological features of embryonic muscles and is thought to originate from activated SCs, whereas alveolar RMS (ARMS) is suggested to originate from uncommitted mesodermal cells or from maturing muscle, and shows poor, if any, muscle differentiation (Qualman et al., 1998). The molecular mechanisms contributing to the high proliferation rate and defective differentiation characterizing RMSs are largely unknown. PAX7 is hyperactive in ARMSs exhibiting PAX7–FOXO1A fusion genes and a widespread (i.e. in >50% of cells) expression of myogenin (Parham and Ellison, 2006; Sebire and Malone, 2003). In addition, PAX7 is upregulated in RMSs lacking PAX3–FOXO1A or PAX7–FOXO1A fusion genes (in general, ERMSs), and ERMSs exhibit varying percentages of myogenin-positive cells (<25% of cells, though) (Sebire and Malone, 2003; Tiffin et al., 2003). However, whether the high PAX7 abundance in ERMS cells is a major cause of their uncontrolled proliferation, and the mechanism of deregulated PAX7 expression in ERMSs, remain to be determined.

The multiligand receptor for advanced glycation end products (RAGE, encoded by AGER), a member of the immunoglobulin superfamily (Bierhaus et al., 2005; Schmidt et al., 2001), is activated by its ligand, high mobility group box 1 protein (HMGB1). RAGE transduces a promyogenic and anti-proliferative signal in myoblasts via activation of a Cdc42/
Rac1–MKK6–p38 MAPK pathway and p38-MAPK-dependent induction of myogenin and the proliferation inhibitor p21\(^{WAF1}\) (also known as CDKN1A) and inactivation of the mitogenic and anti-myogenic kinases, ERK1/2 and JNK (Sorci et al., 2004; Riuzzi et al., 2006). RAGE is expressed in myoblast cell lines and in immature myofibers during prenatal development and the first few postnatal days, and is no longer expressed thereafter (Sorci et al., 2004). However, upon acute injury of adult muscles RAGE becomes re-expressed in activated SCs, proliferating myoblasts and myocytes, and repressed upon completion of regeneration (Riuzzi et al., 2012). We have previously reported that (Riuzzi et al., 2012) HMGB1 and RAGE repress PAX7 transcription in differentiating myoblasts via p38-MAPK-dependent induction of myogenin, and \(Ager^{−/−}\) myoblasts exhibit higher PAX7 expression levels, enhanced proliferation and defective differentiation compared with wild-type (WT) myoblasts. However, we also found that ectopic expression of \(AGER\) in \(Ager^{−/−}\) myoblasts rescues their myogenic potential in part and, compared with WT mice, \(Ager^{−/−}\) mice show delayed regeneration upon acute muscle injury and absence of \(Ager\) results in elevated asymmetric division of primary myoblasts. Thus, RAGE might play a physiological role in muscle regeneration, modulating PAX7 expression, reducing myoblast proliferation and accelerating myogenic differentiation, and regulating SC homeostasis; the premature repression of RAGE in myoblasts and/or inability of myoblasts to upregulate RAGE might therefore result in high PAX7 levels and unrestricted proliferation.

Functional inactivation of RAGE in myoblasts via stable overexpression of a signaling-deficient RAGE mutant resulted in increased proliferation, migration and invasiveness, decreased apoptosis and differentiation, and tumor formation in vivo (Riuzzi et al., 2006). Conversely, enforced expression of full-length \(AGER\) in the ERMS cell line TE671 [which does not express RAGE, the early myogenic differentiation marker myogenin, or the late myogenic differentiation marker, myosin heavy chain (MyHC)] in differentiation medium, results in reduced proliferation, migration and invasiveness, reduced incidence, volume and tumor formation in vivo, and increased apoptosis and myogenic potential, compared with controls (Riuzzi et al., 2007). Taken together, these data suggest that repression of expression of RAGE and/or its functional inactivation at certain stages of adult myogenesis might contribute to rhabdomyosarcomagenesis.

In the present work, we examined whether the high PAX7 abundance that characterizes ERMSs is causally related to their high proliferation rate, and whether low or absent RAGE activity in ERMS cells is correlated with elevated PAX7 levels. We show that in human ERMSs, RAGE expression is low or absent, PAX7 supports ERMS cell proliferation by reducing MyoD levels, and low or absent RAGE activity in ERMS cells causes defective regulation of PAX7 expression thus potentially leading to rhabdomyosarcomagenesis.

RESULTS

Relationships between RAGE and PAX7 expression in ERMSs

We found an inverse relationship between the expression of PAX7 and that of RAGE in tumors generated by inoculation of mice with WT TE671 (TE671/WT) cells or TE671 cells stably transfected with human \(AGER\) (TE671/RAGE cells) (Riuzzi et al., 2006) (Fig. 1A). By examining a panel of 31 human ERMSs for PAX7 and RAGE expression, we found that 14 tumors (~45%) were PAX7+/RAGE−, with the vast majority of them showing no RAGE+ cells; five tumors (~16%) were PAX7+/RAGE+; five (~16%) were PAX7+/RAGE−, with a higher intensity of PAX7+ RAGE− cells in four of them and a higher percentage of RAGE+ cells in one of them; and seven tumors (~23%) were PAX7−/RAGE− (Fig. 1B; supplementary material Fig. S1A). These results pointed to a tendency, although not statistically significant...
(P=0.32; r=-0.19), to an inverse relationship between PAX7 and RAGE. However, in ERMSs with a relatively high RAGE score, PAX7 and myogenin were mutually exclusive, whereas PAX7 and myogenin colocalized in ERMSs with a low RAGE score (supplementary material Fig. S1B). In addition, a tendency, although not statistically significant (P=0.43; r=+0.36), to a positive correlation between PAX7 and RAGE was observed in seven ARMSs lacking PAX–FOXO1A fusion genes, and in six ARMSs showing a PAX3–FOXO1A fusion gene (P=0.18; r=-0.63). In these latter ARMSs, PAX7 was absent, in agreement with the observation that the expression of WT RAGE is downregulated by PAX3–FOXO1A in ARMSs (Tomescu et al., 2004) (Fig. 1C). However, 21 AGER splicing variants have been reported in humans (Kalea et al., 2011). Thus, by the present experimental approach no information could be obtained regarding the RAGE variant(s) we detected. In addition, because we used an anti-RAGE antibody specific to the RAGE V domain, two potential RAGE forms could have been detected in our study, namely full-length RAGE and the signaling-deficient eRAGE (a RAGE form lacking the cytoplasmic and transducing domain). Analogously, the RAGE form lacking the V domain (thus incapable of signaling) escaped detection.

Relationships among the expression levels of RAGE, myogenin, MyoD and PAX7 in ERMS cell lines

We further analyzed the tumors obtained by in vivo inoculation of TE671/WT and TE671/RAGE cells (Riuatti et al., 2007) for expression of myogenin, RAGE, PAX7 and the proliferation marker Ki67. Differently from TE671/WT tumors, in TE671/RAGE masses we found a positive relationship between RAGE and myogenin (supplementary material Fig. S2A), an inverse relationship between PAX7 and myogenin (supplementary material Fig. S2B), and an inverse relationship between myogenin and Ki67, with myogenin and Ki67 being mutually exclusive (supplementary material Fig. S2C). Because the results in Fig. 1B and supplementary material Fig. S1 pointed to a variability of RAGE expression and a tendency to low RAGE expression in ERMSs, and given the inverse relationship between RAGE and PAX7 in TE671/RAGE tumors (Fig. 1A; supplementary material Fig. S2), we reasoned that in ERMS cells PAX7 levels might impact upon RAGE levels and/or the inability of ERMS cells to express relatively high RAGE amounts might account for PAX7 accumulation and unrestricted proliferation. To test this possibility, we firstly performed correlational analyses of RAGE, PAX7, myogenin and MyoD levels in TE671/WT and TE671/RAGE cells. Compared with cells in growth medium, TE671/WT cells in differentiation medium expressed larger PAX7 amounts and similar MyoD amounts, whereas no RAGE, myogenin or MyHC was detected in either growth medium or differentiation medium (Fig. 2A,B). Compared with TE671/WT cells, TE671/RAGE cells exhibited significantly lower PAX7 levels in growth medium and even lower levels in differentiation medium, expression of myogenin in growth medium and, to a larger extent, in differentiation medium, expression of MyHC in differentiation medium, and enhanced expression of MyoD in differentiation medium (Fig. 2A,B). Essentially all TE671/WT cells expressed PAX7 in growth medium and differentiation medium and virtually no RAGE or myogenin in growth medium (supplementary material Fig. S3A,B). Cultivation of TE671/WT cells for 48 h in differentiation medium resulted in the expression and colocalization of myogenin and PAX7 in ~20% of cells, whereas in these same conditions the vast majority of TE671/RAGE cells were myogenin-positive and only the few PAX7+ cells were myogenin negative (supplementary material Fig. S3B). Thus, TE671/RAGE cells show an inverse relationship between myogenin and MyoD levels and PAX7 levels. The ERMS cell lines, RD, RD12 and RD18, each expressed some RAGE and myogenin in growth medium and higher RAGE and myogenin levels in differentiation medium compared with growth medium (Fig. 2C,D). However, differently from TE671/RAGE cells but similarly to TE671/WT cells, RD, RD12 and RD18 cells showed increased PAX7 levels, no changes in MyoD levels and no MyHC expression in differentiation medium compared with growth medium (Fig. 2C,D), and much lower RAGE levels than did TE671/RAGE cells in differentiation medium (Fig. 2E). In growth medium, all RD, RD12 and RD18 cells expressed PAX7 and a minority of them also expressed myogenin (supplementary material Fig. S3). After 48 h in differentiation medium, the percentage of myogenin-positive RD, RD12 and RD18 cells increased, and myogenin colocalized with PAX7 in most cells (supplementary material Fig. S3). Thus, inability of ERMS cells to express relatively high RAGE levels in differentiation medium correlates positively with their high PAX7 levels.

Similar to TE671/RAGE cells, in C2C12 myoblasts and SCs from human young subjects (hu-SCs) (Beccafico et al., 2011), levels of PAX7 decreased and those of RAGE, MyoD, myogenin and MyHC increased in differentiation medium compared with growth medium (Fig. 2F). Thus, forced expression of RAGE in TE671/WT cells partially reverses their phenotype, making these cells similar to C2C12 myoblasts and hu-SCs in terms of ability to downregulate PAX7 and upregulate MyoD, myogenin and MyHC in differentiation medium. In addition, among the ERMS cell lines examined here, only in TE671/RAGE cells did the extent of RAGE expression correlate negatively with PAX7 expression and positively with MyoD, myogenin and MyHC expression. Thus, RAGE levels and/or the intensity of RAGE signaling might regulate PAX7, MyoD and myogenin levels in ERMS cells.

RAGE engagement in myoblasts results in activation of p38 MAPKs (Sorci et al., 2004), which are kinases required for expression of myogenin and myogenic differentiation (Lluis et al., 2006), and inhibition of p38 MAPK results in inhibition of myogenin expression in TE671/RAGE cells (Riuatti et al., 2007). We found that inhibition of p38 MAPK by the specific inhibitor, SB203580, did not affect PAX7 abundance in TE671/WT cells in differentiation medium, whereas it led to a substantial increase in PAX7 levels in TE671/RAGE cells (Fig. 2G) similar to hu-SCs (unpublished results) and C2C12 myoblasts (Riuatti et al., 2012). In addition, TE671/WT, RD, RD12 and RD18 cells in differentiation medium exhibited a similar proliferation rate to cells in growth medium, in contrast to TE671/RAGE cells which showed a lower proliferation rate in growth medium with a further decrease in differentiation medium compared with TE671/WT cells (Fig. 2H). Thus, in ERMS cells low or absent RAGE signaling might account for defective regulation of PAX7, MyoD and myogenin, and unrestricted proliferation, whereas activation of a p38–MAPK–myogenin axis via enforced AGER expression reduces PAX7 abundance and proliferation. To investigate this possibility in detail, we analyzed the effects of transient transfection of ERMS cells with increasing doses of either AGER or Myog. We performed these experiments in growth medium in order to avoid alterations generated by the low levels of serum mitogens in differentiation medium.
Dual regulation of PAX7 expression by the RAGE-myogenin axis

Transfection of TE671/WT cells with increasing AGER doses up to 6 μg resulted in increased MYOG, MYOD1 and PAX7 levels, whereas at 9 μg ectopic AGER further increased MYOD1 levels and dramatically reduced PAX7 levels (Fig. 3A). In these conditions, ectopic AGER (1) dose-dependently increased myogenin levels, (2) increased MyoD levels at ≥6 μg, (3) at up to 6 μg increased PAX7 levels, and (4) increased MyHC levels and robustly decreased PAX7 levels at 9 μg (Fig. 3B). Transfection of RD cells with AGER also resulted in a dose-dependent increase in myogenin, MyoD and MyHC levels, and in increased PAX7 levels at 1 μg, and significantly decreased PAX7 levels at higher doses (Fig. 3A). Incidentally, the amount of RAGE detected in TE671/WT cells upon transfection with 9 μg AGER was comparable to that detected in normal myoblasts after 48 h in differentiation medium (supplementary material Fig. S4A). Thus, ectopic AGER exerted a dual effect on PAX7 expression in ERMS cells, that is, an increase at low transfection doses and a dramatic decrease at high transfection doses, and a dose-dependent increase in MyoD and myogenin expression.
Transfection of TE671/WT cells with Myog caused a dose-dependent increase in PAX7 levels up to 6 µg and a dramatic decrease at 9 µg (Fig. 4A). Transient transfection of TE671/WT cells with Myog for 48 h and then with pGL3B/PAX7(−4800)-luc for 24 h also resulted in enhanced and reduced transcription of PAX7 at 6 µg and 9 µg Myog, respectively (Fig. 4C). However, PAX7 levels did not change at 1 µg Myog, dose-dependently increased at 3 and 6 µg Myog, and remarkably decreased at 9 µg (Fig. 4B). Incidentally, the amount of myogenin detected in TE671/WT cells upon transfection with 9 µg Myog was similar to that detected in normal myoblasts after 48 h in differentiation medium (supplementary material Fig. S4B). By contrast, ectopic Myog increased MYOD1 levels, with a similar efficiency from 1 to 9 µg (Fig. 4A), and MyoD levels dose-dependently (Fig. 4B). The different expression patterns of PAX7 and PAX7 and of MYOD1 and MyoD in Myog-transfected TE671 cells suggested that there were transcriptional, post-transcriptional and/or post-translation events (see below). Transfection of RD cells with increasing doses of Myog resulted in similar expression patterns of PAX7 and MyoD mRNAs and proteins to TE671/WT cells (Fig. 4A,B). Double immunofluorescence analyses documented an enhanced PAX7 expression in TE671/WT cells in terms of PAX7 fluorescence intensity in individual cells upon transfection with 6 µg Myog, and a reduced PAX7 expression in TE671/WT cells in terms of numbers of PAX7+ cells and PAX7 fluorescence intensity following transfection with 9 µg Myog, and a reduction of the number of proliferating (Ki67+) TE671/WT cells following transfection with either Myog dose (supplementary material Fig. S5A). These cells also displayed a colocalization of PAX7 and myogenin upon transfection with 6 µg Myog and a mutually exclusive cellular localization of PAX7 and myogenin upon transfection with 9 µg Myog (supplementary material Fig. S5B), and an increase in MyoD expression and co-localization of MyoD and myogenin upon transfection with Myog (supplementary material Fig. S5C). Finally, there was also a mutually exclusive localization of myogenin and Ki67 upon transfection with Myog (supplementary material Fig. S5D). Transfection of hu-SCs (Fig. 4A,B) with 1 µg Myog resulted in increased PAX7 levels, whereas transfection with 3 µg Myog was sufficient to reduce PAX7 expression. However, ectopic Myog dose-dependently increased MyoD mRNA and protein (Fig. 4A,B). Importantly, in the ERMS cell lines investigated here, Myog at the dose of 9 µg (Fig. 4C) but not smaller doses (data not shown), induced MyHC expression in growth medium coincidently with a substantial decrease in PAX7 levels. Noteworthy, ectopic Myog did not change the levels of AGER and RAGE in TE671/WT and RD cells, whereas it dose-dependently increased them in hu-SCs (Fig. 4A,B) (see Discussion).

Collectively, these results showed an unprecedented relationship between the RAGE–myogenin axis and PAX7 in ERMS cells and hu-SCs: within a certain range of abundance, ectopic AGER or Myog increased PAX7 levels, whereas high
AGER or Myog reduced PAX7 levels; however, AGER and Myog upregulated MyoD levels at any dose. Thus, in ERMS cells absent or low RAGE levels and/or signaling might be an important cause of defective regulation of PAX7 levels and of low myogenin and MyoD levels. Significantly, a dual regulation of PAX7 depending on the AGER or Myog dose also occurs in hu-SCs, but here the dose–response curve is shifted to the left.

**Ectopic AGER or Myog reduces ERMS cell proliferation via MYOD1 induction**

Transfection of TE671/WT and RD cells with ≥ 3 μg Myog also resulted in a robust reduction of the proliferation marker, cyclin D1, and of cell proliferation (Fig. 4B; supplementary material Fig. S5A), whereas a smaller Myog dose was sufficient to reduce cyclin D1 levels in hu-SCs (Fig. 4B). Transfection of TE671/WT with AGER also resulted in a dose-dependent reduction of cyclin D1 (Fig. 3B). Thus, ectopic AGER or Myog dramatically reduced ERMS cell proliferation in coincidence with either high or low PAX7 levels.

We asked how ectopic AGER or Myog might inhibit ERMS cell proliferation in growth medium irrespective of PAX7 levels. As shown in Fig. 2A,B, compared with TE671/RAGE cells, TE671/WT cells in growth medium and differentiation medium expressed lower amounts of MyoD, which is responsible for proliferation arrest in cells of the myogenic lineage (Crescenzi
et al., 1990; Guo et al., 1995; Halevy et al., 1995; Puri et al., 1997; Sorrentino et al., 1990) and for activation of muscle-specific gene transcription (Asakura et al., 2007; Weintraub et al., 1991). Levels of MyoD in TE671/WT and RD cells increased dose-dependently upon transfection with either AGER or Myog pointing to RAGE- and/or myogenin-dependent upregulation of MyoD (Fig. 3A,B; Fig. 4A,B, respectively). However, given that a reduction of cyclin D1 levels and cell proliferation was seen irrespective of PAX7 levels, provided myogenin and/or MyoD were expressed in relatively high amounts, PAX7 might hardly be directly responsible for this or, alternatively, PAX7 might indirectly regulate TE671 cell proliferation. Incidentally, transfection of TE671/WT and RD cells with either PAX7 or AGER resulted in a dose-dependent increase in apoptosis (supplementary material Fig. S5E,F, respectively) in coincidence with upregulation of MyoD (Fig. 3A,B; Fig. 4A,B), in accordance with the reported ability of MyoD to downregulate anti-apoptotic genes (Asakura et al., 2007).

Because the MYOD1 promoter contains 17 PAX7 recognition sites and eight myogenin recognition sites (PatchTM public 1.0 pattern search for transcription-factor-binding sites) we reasoned that when expressed and within a certain range of abundance in TE671/WT cells, myogenin might upregulate both MyoD and PAX7, and that this upregulated MyoD might inhibit cell proliferation. In addition, given the reported ability of PAX7 to promote myogenin and MyoD proteasomal degradation and vice versa (Olguin et al., 2007), myogenin and MyoD might compete for PAX7-dependent degradation, such that in the presence of high levels of myogenin less MyoD becomes degraded. The resulting high MyoD levels would lead to a reduction in TE671/WT cell proliferation. To address this hypothesis we knocked down PAX7 by RNA interference in TE671/WT and RD cells and, conversely, transiently transfected these cell types with Myod1 and huSCs with PAX7, as described below.

**PAX7 reduces MyoD and myogenin levels in ERMS cells**

Levels of PAX7 in ERMS cells were much higher than in TE671/RAGE cells and hu-SCs (supplementary material Fig. S6). Compared with controls, knockdown of PAX7 in TE671 and RD cells resulted in no significant changes in MYOG or MYOD1 levels (Fig. 5A), suggesting that PAX7 might not affect MYOG or MYOD1 transcription in ERMS cells, and that the enhancement of MYOD1 levels in coincidence with RAGE- or myogenin-induced upregulation of PAX7 (Fig. 3A,B; Fig. 4A,B) was dependent on myogenin but not PAX7. However, a substantial increase in MyoD and myogenin and apoptosis, and a decrease in cyclin D1 and the percentage of Ki67+ cells occurred in PAX7 siRNA-treated ERMS cells compared with controls (Fig. 5B–D). The high MyoD and myogenin levels generated by PAX7 knockdown in ERMS cells could depend on reduced MyoD and myogenin degradation as a consequence of decreased PAX7 (Olguin et al., 2007), and be an important cause of reduced proliferation and enhanced apoptosis. Indeed, treatment of TE671/WT cells with the proteasomal inhibitor MG132 resulted in higher MyoD and myogenin (and PAX7) levels compared with their respective controls (Fig. 5E). Importantly, knockdown of PAX7 resulted in a small but significant increase in AGER levels and detection of RAGE in TE671/WT cells, and in increased levels of RAGE mRNA and protein in RD cells, suggesting that PAX7 represses RAGE expression in ERMS cells (Fig. 5A,B), in accordance with the notion that the AGER promoter contains PAX recognition sites (Riuuzzi et al., 2012).

By contrast, transfection of hu-SCs with increasing PAX7 doses up to 6 μg resulted in increased myogenin (mRNA and protein) and cyclin D1 levels (Fig. 5F,G), whereas upon transfection with 9 μg PAX7 a substantial decrease in cyclin D1 (Fig. 5F) was detected along with remarkably low myogenin mRNA and protein levels relative to the control, as investigated by real-time PCR (Fig. 5F), western blotting (Fig. 5G) and analysis of myogenin-luc reporter gene expression (Fig. 5H). Interestingly, recent work has shown that transfection of C2C12 myoblasts with Pax7 or dominant-negative Pax7 results in an enhanced and reduced transcription of Myog, respectively, compared with controls (Calhabeu et al., 2013). We show here that up to a certain level, in hu-SCs, PAX7 normally upregulates myogenin, whereas above a critical level of abundance, PAX7 downregulates myogenin (Fig. 5F–H). Thus, PAX7 and myogenin reciprocally regulate their expression levels in a bimorphic manner in normal myoblasts. In addition, in hu-SCs ectopic PAX7 decreased RAGE mRNA and protein dose dependently (Fig. 5F,G), increased MYOD1 levels at 1 and 3 μg, and substantially decreased it at 9 μg, and decreased MyoD dose dependently (Fig. 5F,G). The dose-dependent decrease in MyoD, as opposed to the biphasic change in MYOD1 detected in PAX7-transfected hu-SCs, likely reflects transcriptional (Fig. 5E) and post-translational (Olguin et al., 2007) regulatory mechanisms.

Collectively, our results suggested that: (1) ERMS cell proliferation and survival require a certain amount of PAX7; (2) PAX7 enhances ERMS cell proliferation by reducing MyoD levels in a proteasome-dependent manner; and (3) low or absent RAGE signaling in ERMS cells alters the relative abundances of PAX7, MyoD and myogenin, with a prevalence of PAX7 over MyoD and myogenin, which leads to enhanced proliferation and defective differentiation, similar to in Ager−/− myoblasts (Riuuzzi et al., 2012).

**MyoD regulates PAX7 and myogenin levels in ERMS cells**

Transient transfection of TE671/WT, RD and hu-SCs with increasing doses of Myod1 resulted in a dose-dependent increase in myogenin and decrease in PAX7, and no significant changes in RAGE (Fig. 6A,B). Transfection with Myod1 also reduced cyclin D1 levels in all the above cell types (Fig. 6B) and proliferation in TE671/WT cells (Fig. 6C) and all other cell types listed above (data not shown). Consistently, transfection of TE671/WT cells with increasing Myod1 doses progressively made Ki67 and MyoD mutually exclusive (Fig. 6C) strongly indicating that low or absent expression of MyoD in ERMS cells is due to their high PAX7 content (see Fig. 5B,F) and is a major cause of elevated proliferation.

**DISCUSSION**

ERMS is a subtype of RMS that has been suggested to originate from activated SCs that have acquired uncontrolled proliferation and inability to terminally differentiate. ERMSs are immunohistochemically identified by a low percentage of myogenin-positive cells (Parham and Ellison, 2006) and show high PAX7 levels (Tiffin et al., 2003). High PAX7 has been linked to uncontrolled proliferation in ERMSs, but no formal evidence for this has been provided thus far. Based on the observation that RAGE signaling in myoblasts inhibits proliferation, stimulates differentiation and represses Pax7 transcription (Riuuzzi et al., 2006; Riuuzzi et al., 2012; Sorci et al., 2004), and that forced expression of RAGE in the TE671
cell line, which does not express RAGE, causes a robust decrease in proliferation, an increase in apoptosis and a partial rescue of their myogenic potential (Riuzzi et al., 2007), we performed a comparative analysis of PAX7 and RAGE expression in a panel of human ERMSs. We found that: (1) 68% of the examined ERMSs were RAGE⁺; (2) a minority (16%) of them expressed PAX7 and RAGE; and (3) RAGE immunostaining in RAGE⁺ ERMSs was never of the highest grade. Based on these and previous results (Riuzzi et al., 2006; Riuzzi et al., 2007; Riuzzi et al., 2012), we used ERMS cell lines to investigate whether the inability to express relatively high RAGE levels might cause accumulation of PAX7 and unrestricted proliferation. Herein, we have shown that in ERMS cell lines: (1) RAGE expression is absent or low, whereas PAX7 levels are high, compared with normal myoblasts; (2) PAX7 reduces the levels of the myoblast proliferation inhibitor and pro-apoptotic factor MyoD, primarily in a proteasome-dependent manner; (3) this event is causally related to the high proliferation rate and low extent of apoptosis; (4) that low or absent RAGE signaling might have an important tumorigenic role by promoting PAX7 accumulation and consequent remarkably low MyoD levels as a result of defective expression of myogenin; and (5) ectopic expression of AGER results in reduced PAX7 abundance and cell proliferation, enhanced apoptosis and expression of myogenic factors.
Several lines of evidence support these conclusions. Firstly, ERMS cells show defective regulation of PAX7, MyoD, myogenin and RAGE when transferred from growth medium into differentiation medium. In fact, relative to the growth medium conditions, in ERMS cells cultivated in differentiation medium PAX7 and myogenin levels increase, whereas no major changes occur in MyoD levels. By contrast, upon transfer from growth medium into differentiation medium, normal myoblasts and TE671/RAGE cells show a significant decrease in PAX7 levels and an increase in myogenin and MyoD levels. In addition, although an increase in the amount of RAGE occurs in some ERMS cell lines following their transfer from growth medium into differentiation medium, RAGE abundance in these cells is much lower than that found in TE671/RAGE cells. The defective regulation of PAX7, myogenin, MyoD and RAGE in ERMS cells correlates positively with their similar proliferation rates in growth medium and differentiation medium.

Secondly, the relatively high myogenin and MyoD levels obtained by either stable (Riuzzi et al., 2007) or transient transfection of ERMS cells with \textit{AGER} are invariably accompanied by a reduced proliferation and enhanced apoptosis. Proliferation is similarly reduced upon transient transfection of ERMS cells with \textit{Myog}. However, both \textit{AGER} and \textit{Myog} exert a dual effect on PAX7 levels in ERMS cells, that is, an increase at relatively low doses and a dramatic decrease at relatively high doses of either transfectant. However, irrespective

Fig. 6. MyoD regulates PAX7 and myogenin levels in ERMS cells. (A,B) TE671/WT, RD cells and hu-SCs were cultivated in growth medium, transfected with increasing doses of \textit{Myod1} for 48 h and subjected to real-time PCR (A) and western blotting (B) for detection of the indicated cDNAs and proteins, respectively. Results are means ± s.d. \((n=3)\). \(*P < 0.05\) compared with control. (C) TE671/WT cells were treated as in A and B and subjected to double immunofluorescence using an anti-MyoD and anti-Ki67 antibody. Scale bars: 50 μm.

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of the dose of transfected AGER or Myog, MyoD levels are upregulated, and this correlates negatively with ERMS proliferation and positively with apoptosis, regardless of PAX7 levels. In addition, when induced by ectopic AGER or Myog, MyoD can repress PAX7 transcription. Whether MyoD represses PAX7 transcription directly or through the prior upregulation of MYOG remains to be established. In addition, whereas in ERMS cells PAX7 does not affect MYOD1 transcription, PAX7 promotes proteasomal degradation of MyoD and myogenin. Thus, it is a RAGE–myogenin–MyoD axis that reduces PAX7 levels and proliferation and increases apoptosis in AGER- or Myog-transfected ERMS cells: in the presence of low or no RAGE, PAX7 accumulates owing to insufficient myogenin and MyoD abundance, with consequent high proliferation, low apoptosis and absence of myogenic differentiation, a scenario recalling the condition seen in cultures of normal myoblasts in growth medium (Fig. 7A). It is noteworthy that PAX7 reduces RAGE expression in ERMS cells and hu-SCs. Also of note is that disruption of MyoD and p21WAF1 activity in rhabdomyosarcoma has been long recognized (Weintraub et al., 1997), and RAGE signaling in myoblasts impacts on p21WAF1 abundance (Riuzzi et al., 2007; Sorci et al., 2004). Characteristically, in human ERMS, only a minor fraction of cells are positive for myogenin (Sebire and Malone, 2003). Our results suggest that this might be because in ERMSs RAGE is not expressed or is expressed at levels insufficient to induce levels of myogenin and MyoD capable of reducing PAX7 abundance.

A dual myogenin-dependent regulation of PAX7 levels also occurs in hu-SCs and appears to be aimed at regulating PAX7, MyoD and myogenin levels. Indeed, when expressed at low levels, myogenin upregulates PAX7 mRNA and protein, and coincidently causes a modest increase in MyoD mRNA and protein. However, in this condition the levels of the proliferation marker cyclin D1 do not change, suggesting that the levels of MyoD are not sufficient to reduce proliferation. Further increases in PAX7 levels in hu-SCs promote the accumulation of myogenin and cyclin D1, while reducing RAGE and MyoD levels. This scenario recalls the one detected during the first 8 h of cultivation of myoblasts in differentiation medium (Fig. 7B), when myoblasts express relatively high PAX7 levels and relatively low myogenin and RAGE levels, yet still proliferate (Riuzzi et al., 2012). Therefore, it is not surprising that we detect a relatively high variability of RAGE and PAX7 expression levels in human ERMSs with a tendency to high PAX7 levels and low or absent RAGE (Fig. 1B), similar to in TE671, RD, RD12 and RD18 cell lines (Fig. 2A–D). RAGE-positive human ERMSs are

Fig. 7. Schematic representation of the proposed mechanism of reciprocal regulation of PAX7, MyoD and myogenin. (A) In proliferating normal myoblasts, RAGE levels are insufficient to induce myogenin. At the relatively high levels found in proliferating myoblasts, PAX7 inhibits RAGE, MyoD and myogenin expression, which results in a high proliferation rate, a low extent of apoptosis and prevention of differentiation. (B) During the first few hours (e.g. 8 h) in differentiation medium, activation of p38 MAPK results in upregulation of myogenin, which in turn upregulates RAGE, PAX7 and MyoD. In this condition, PAX7 promotes accumulation of myogenin and prevents excessive RAGE and MyoD expression, thus transiently sustaining myoblast proliferation. (C) At a later differentiation time (e.g. 48 h), the RAGE- and p38 MAPK-dependent increase in myogenin results in repression of PAX7 expression and upregulation of MyoD leading to reduction of proliferation and terminal differentiation. (D) In ERMS cells, low or absent RAGE signaling translates into high PAX7 levels because of low or no myogenin and MyoD expression, which in turn results in unrestricted proliferation, a low extent of apoptosis and no differentiation. Induction of RAGE in ERMS cells results in expression of myogenin and MyoD levels, such as to cause repression of PAX7 expression and PAX7 proteasomal degradation, ultimately leading to reduction of proliferation, enhancement of apoptosis and myogenic differentiation. In A–D, thick lines represent prevailing activities brought about by the molecules in bold, leading to prevalent biological effects (in bold), whereas thin lines represent low-intensity or no activities. Lines of intermediate width in B indicate that in early differentiating myoblasts the shown molecules exert intermediate reciprocal effects, so that proliferating cells coexist with differentiating cells. Upwards and downwards arrows represent enhancement and reduction of biological effects, respectively.
likely to express relatively low RAGE levels, that is, much the same as normal myoblasts early during the differentiation phase, and are thus insufficient to repress ERMS cell proliferation.

However, further increases in myogenin levels (as obtained by transfection of hu-SCs with 3 μg Myog) cause a dramatic decrease in PAX7 levels coincidently with an increase in MyoD and RAGE and a likewise dramatic decrease in cyclin D1 levels. This latter scenario recalls the one detected at 48 h of cultivation of myoblasts in differentiation medium (Fig. 7C), when under the action of a RAGE–p38-MAPK axis myoblasts (1) express high myogenin, MyoD and RAGE levels and low if any PAX7 levels, (2) significantly reduce their proliferation rate, and (3) differentiate (Riuzzi et al., 2012). Collectively, our results suggest that PAX7, MyoD and myogenin dose-dependently regulate each other’s levels and that the relative abundances of PAX7, MyoD and myogenin determine the final destiny of the myoblasts (i.e. proliferation, apoptosis or differentiation). Our results support a model in which high-intensity RAGE signaling increases MyoD and myogenin levels in normal myoblasts with ensuing inhibition of PAX7 transcription and elevated PAX7 proteasomal degradation; the reduction of PAX7 levels translates into augmented levels of the myoblast proliferation inhibitor, MyoD, and of myogenin leading to enhanced apoptosis, reduced proliferation and differentiation (Fig. 7C). In this scenario, absence or blockade of RAGE expression and/or activity results in the accumulation of PAX7 in myoblasts owing to insufficient MyoD and myogenin abundance with a resulting high proliferation rate, low apoptosis and defective differentiation (Fig. 7A), as observed in myoblasts stably expressing a signaling-deficient RAGE mutant (Riuzzi et al., 2006) and in Ager−/− myoblasts (Riuzzi et al., 2012). By the same reasoning, as we first describe here, absence of RAGE expression or low RAGE activity in ERMS cells may result in alteration of the PAX7–myogenin and PAX7–MyoD interplay owing to absent or reduced myogenin and/or MyoD expression (Fig. 7D), ultimately leading to high PAX7 levels, low apoptosis, no differentiation and unrestricted proliferation. Intriguingly, ectopic Myog did not induce or upregulate RAGE expression in ERMS cells in growth medium, in contrast to the myogenin-dependent upregulation of RAGE expression in C2C12 myoblasts (Riuzzi et al., 2012) and hu-SCs (Fig. 4A,B). This points to a complex regulation of RAGE expression in cells of the myogenic lineage; perhaps ERMS cells lack a factor(s) required for myogenin-dependent RAGE expression, which might be a consequence of SC neoplastic transformation and, in turn, an important cause of ERMS genesis. However, absence of RAGE might not be causative of RMS formation per se because inoculation of Ager−/− myoblasts into NOD-SCID mice does not result in tumor formation and because no RMS formation can be observed in Ager−/− mice irrespective of their age (up to 2 years after birth) (Sara Chiappalupi, F.R., Stefania Fulle, R.D. and G.S.). Thus, absence or reduced activity of RAGE is likely to be accompanied by other factors/conditions for rhabdomyosarcomagenesis.

Conversely, because 16% of the ERMSs examined here were PAX7+/RAGE+ and another 16% were PAX7+/RAGE−, the presence of RAGE does not prevent ERMS genesis per se. Indeed, RD, RD12 and RD18 ERMS cells do express RAGE in growth medium and upregulate RAGE levels in differentiation medium. However, once transferred into differentiation medium these ERMS cell lines also show upregulated PAX7 and myogenin in the presence of unchanged MyoD levels and RAGE levels that are much smaller than those found in, for example, TE671/RAGE cells. However, transfection of RD cells with RAGE results in a significant reduction of proliferation and increase in apoptosis and in myogenic differentiation. Thus, in those human ERMSs that express RAGE, the levels and/or signaling intensity of RAGE might not be sufficient to efficiently activate p38 MAPK, the master kinase responsible for myoblast proliferation arrest and myogenic differentiation. In this regard, defective p38 MAPK activation has been documented in several RMS cell lines, and enforced induction of p38 MAPK activity (Puri et al., 2000) or RAGE (Riuzzi et al., 2012, and the present study) leads to growth arrest and terminal differentiation. In addition, it should be stressed that several splice variants of human AGER exist, including the variant encoding the signaling-deficient sRAGE (Kalea et al., 2011), which is detected by the anti-RAGE antibody used in the present study. Thus, not all the RAGE-expressing ERMSs analyzed in Fig. 1B might express functional RAGE, which reinforces the conclusion that inability of ERMSs to express sufficient RAGE (owing in part to their high PAX7 levels) might play an important tumorigenic role. Aside from low RAGE levels and/or signaling in ERMSs, other factors and/or conditions might cause ERMSs to express high PAX7 levels. For example, altered expression levels of N-cadherin (Charrasse et al., 2004) or Cdo (Wegorzewska et al., 2003) in ERMSs might contribute to high PAX7 levels because signaling of N-cadherin (Lovett et al., 2006) and Cdo (Takeasu et al., 2006) impacts upon p38 MAPK.

In conclusion, we have shown that (1) in ERMS cells, a mechanistic link exists between low or absent RAGE signaling and high PAX7 expression levels, low MyoD and myogenin levels and unrestricted proliferation; and (2) conditions leading to expression of either myogenin or full-length RAGE in ERMSs might exert anti-tumor effects. It is known that ERMSs characteristically exhibit low percentages of myogenin-positive cells (Parham and Ellison, 2006; Sebire and Malone, 2003), and increases in the percentages of cells exhibiting markers of myogenic differentiation in ERMSs following chemotherapy and/or radiotherapy are taken as an index of efficacious therapy (Smith et al., 2002). Thus, we propose that (1) inability to express relatively high RAGE levels and/or defective RAGE signaling might substantially contribute to ERMS genesis via deregulation of PAX7 expression; (2) that RAGE can be a potential therapeutic target in ERMSs; and (3) that analysis of RAGE expression in ERMSs might represent a tool for ERMS sub-classification and, potentially, for prognostic use.

MATERIALS AND METHODS

Cell culture

Human ERMS TE671 and RD cells were obtained from the ATCC (CRL-8805 and CCL-136, respectively). RD12 and RD18 ERMS cells were provided by Pier-Luigi Lollini (University of Bologna, Bologna, Italy) (Lollini et al., 1991). TE671/RAGE cells were obtained by stable transfection of TE671 cells with full-length human AGER (Riuzzi et al., 2007). Hu-SCs were provided by Stefania Fulle (University of Chieti-Pescara, Chieti, Italy). Where specified, cells were treated with the p38 MAPK inhibitor SB203580 (5 μM, Calbiochem), or the proteasomal inhibitor MG132 (25 μM, Sigma Aldrich). TE671, RD, RD12, RD18 and TE671/RAGE cells were expanded, and aliquots were frozen in liquid nitrogen; each aliquot was cultured for no more than 10 passages. TE671, RD, RD12, RD18 and TE671/RAGE cells were cultivated in high-glucose Dulbecco’s modified Eagle’s medium (HG-DMEM) supplemented with 5% fetal bovine serum (FBS) (Invitrogen), 100 U/ml penicillin, and 100 μg/ml streptomycin (growth medium) in the absence (TE671, RD, RD12 and RD18 cells) or presence (TE671/RAGE cells) of geneticin. Hu-SCs were purified from the Vastus lateralis.
muscle biopsies of young healthy untrained men, characterized and grown in HAM’s F-10 supplemented with 20% FBS (Beccafico et al., 2011). Subjects underwent voluntary biopsy within a protocol approved by the Ethics Committee for Biomedical Research, University of Chieti-Pescara, Chieti, Italy (Prot. 1884 COET). All cell types were cultivated in an H2O2-saturated, 5% CO2 atmosphere at 37°C. Where required, sub-confluent cultures were shifted to antibiotic-containing DMEM supplemented with 2% horse serum (differentiation medium).

**Reverse transcription PCR and real-time PCR**

Total RNA was extracted using the TRIZol reagent (Invitrogen) according to the manufacturer’s instructions. Denaturation was performed at 95°C for 30 sec, annealing for 1 min and extension at 72°C for 30 sec. See supplementary material Table S1 for primer sets and annealing temperatures. DNA was mixed with Phire™ HotStart DNA Polymerase (Finnzymes) in a reaction volume of 20 μl. To detect AGER, mouse Ager, PAX7, MYOD, mouse Myog, MYOD1 and mouse Myd1 by real-time PCR we used the primer sets in supplementary material Table S2 (denaturation at 95°C for 15 s, annealing at 60°C for 20 s and extension at 72°C for 20 s). DNA was mixed with HOT TAQ EvaGreen® qPCR Mix Plus (ROX) ready-to-use solution (Solis BioDyne) in a reaction volume of 20 μl. Reaction mixtures were incubated in a thermocycler (Stratagene Mx3000P). Amplification curve plotting and calculation of Ct values were performed by dedicated software. The ACTB gene was used as an internal standard.

**Western blotting**

Equal amounts of protein were separated by SDS-PAGE. Cells were lysed, and cell lysates were subjected to western blotting as described previously (Sorci et al., 2003). The following antibodies were used: polyclonal anti-AGER (1:1000, Santa Cruz Biotechnology), monoclonal anti-PAX7 (1:500, R&D Systems), monoclonal anti-MyoD (1:500, Santa Cruz Biotechnology), monoclonal anti-myogenin (1:1000, BD Biosciences), polyclonal anti-cyclin D1 (1:500, Santa Cruz Biotechnology), monoclonal anti-MyHC (1:500, Monosan), polyclonal anti-phosphorylated-p38-MAPK (Thr180/Tyr182, D3F9, 1:1000; Cell Signaling Technology), polyclonal anti-p38-MAPK (1:1000, Cell Signaling Technology), and monoclonal anti-α-tubulin (1:10,000, Sigma Aldrich). The immune reaction was developed by enhanced chemiluminescence (SuperSignal West Femto Maximum or SuperSignal West Pico, both from Pierce).

**Transfection and gene knockdown**

Transient transfections were carried out using jetPEI™ (Polyplus Transfection) as recommended by the manufacturer. Briefly, cells were transfected with 1, 3, 6 or 9 μg/well of pcDNA3/human RAGE, pEMSV- mouse myogenin, pcDNA3/human PAX7d, pESMV- mouse MyoD or empty vector. When required, empty vector was used as carrier DNA to transfet 9 μg of total DNA in every case. Subconfluent cells were transfected with control siRNA-A (Santa Cruz Biotechnology) or PAX7 siRNA(hu) (Santa Cruz Biotechnology) using Interferin™ (Polyplus Transfection) according to the manufacturer’s instructions. After cultivation, as described in the figure legends, the cells were subjected to either real-time PCR or western blotting. For luciferase reporter assay, cells were transfected with a luciferase reporter vector under the control of the −4800 bp region of the human PAX7 promoter (pGL3b −4800) (Mummert et al., 2000) or the myogenin-luc reporter gene. After 24 h the cells were harvested to measure luciferase activity by the luciferase assay system (Promega).

**Cell proliferation and apoptosis**

Cell proliferation was measured by BrdU incorporation assay or counts of Ki67 cells. Apoptosis was measured as described previously (Riuuzzi et al., 2006).

**Immunohistochemistry and immunofluorescence**

ERMS and ARMS specimens were subjected to immunohistochemistry (Riuuzzi et al., 2006) using a monoclonal anti-PAX7 (1:50, R&D Systems) or a polyclonal anti-RAGE (1:50, Santa Cruz Biotechnology) antibody and evaluated by light microscopy, using a 10x magnification objective. A semi-quantitative approach was used to generate a score for RAGE and PAX7 expression as follows: a value assigned for the percentage of positive cells/slice (0, 0–10%; 1, 11–20%; and so on) was multiplied by the dominant intensity pattern of staining (1, weak; 2, moderate; 3, intense); therefore, the overall score ranged between 0 (i.e. 0–10% of the cells showing any staining) to 27 (i.e. 91–100% of the cells showing intense staining). Specimens with scores <5 (i.e. ≤50% of the cells with weak positivity, or ≤30% of the cells with moderate positivity) were classified as negative. Specimens with low levels of RAGE and PAX7 were reevaluated using a 40x objective.

Double immunofluorescence on tissue slices was performed as above except that PBS, pH 7.4, instead of TBS and a different blocking buffer (BB; 0.4% Triton-X-100, 10% donkey serum, 1% BSA in PBS) were used. The primary antibodies (1:20 in BB) were: goat polyclonal anti-RAGE (Santa Cruz Biotechnology), mouse monoclonal anti-PAX7 (R&D Systems), mouse monoclonal anti-myogenin (Santa Cruz Biotechnology), mouse monoclonal anti-MyoD (Santa Cruz Biotechnology), and rabbit polyclonal anti-Ki67 (Santa Cruz Biotechnology). The secondary antibodies were donkey Alexa-Fluor-488-conjugated anti-mouse-IgG (Invitrogen), donkey Alexa-Fluor-594-conjugated anti-goat-IgG (Invitrogen) and goat rhodamine-conjugated anti-rabbit-IgG (Sigma Aldrich). For double-immunofluorescence of cultured cells, cells were grown on glass coverslips, fixed in 4% paraformaldehyde in PBS and processed with BB, primary and secondary antibodies as above. Nuclei were counterstained with DAPI. After rinsing, samples were mounted with fluorescent mounting medium (Dako Corporation) and viewed in an epifluorescence microscope (Leica DMRB) equipped with a digital camera.

**Statistical analysis**

Each experiment was repeated at least three times. Representative experiments are shown unless stated otherwise. The data were subjected to analysis of variance (ANOVA) with SNK post hoc analysis using a statistical software package (GraphPad Prism version 4.00, GraphPad). ERMS scores were analyzed using the statistical software Stata12.

**Acknowledgements**

We wish to thank Heikki Rauvala (Helsinki, Finland) for providing AGER expression vector, Bradley Ohlin (Boulder, CO) for providing PAX7 and Myog expression vectors, Eyal Bengal (Haifa, Israel) for providing the myogenin-luc construct, Pier Lorenzo Puri (La Jolla, CA) for providing Myod1 expression vector, Beat Schäfer (Zurich, Switzerland) for providing the pGL3b-4800 PAX7-luc construct. We wish to thank Prof. Fabrizio Stracci, Perugia University, for statistical analysis of ERMS scores.

**Competing interests**

The authors declare no competing interests.

**Author contributions**

F.R., G.S. and R.D. conceived and designed the experiments. F.R., R.S. and G.S. performed the experiments. F.R., G.S. and R.D. analyzed the data. A.S., R.A. and V.N. provided human ERMSs. G.S. and R.D. wrote the paper.

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**Supplementary material**

Supplementary material online at http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.136259/-/DC1

**References**


Fig. S1. Inverse relationship between PAX7 expression and RAGE expression in human ERMSs. (A) Shown are examples of ERMSs exhibiting high and low expression levels of PAX7 and RAGE, respectively (ERMS#1), intermediate levels of expression of PAX7 and RAGE (ERMS#13), and low and high expression levels of PAX7 and RAGE, respectively (ERMS#23). (B) Human ERMSs with RAGE scores 8 (ERMS#18) and 1 (ERMS#6), respectively, were subjected to double immunofluorescence using an anti-PAX7 (green) and anti-myogenin (red) antibody. The RAGE score 8 ERMS (ERMS#18) shows a relatively small number of PAX7+ and myogenin cells and no PAX7 and myogenin colocalization, whereas in RAGE score 1 ERMS (ERMS#6) nearly all cells are PAX7+ and a large fraction of them are myogenin+ with colocalization of PAX7 and myogenin. (Bars in A and B = 200 µm).
Fig. S2. Immunofluorescence analysis of PAX7, RAGE, myogenin and Ki67 in tumor masses obtained by in vivo inoculation of TE671/WT and TE671/RAGE cells. (A) Tumor masses were subjected to double immunofluorescence using an anti-RAGE (red) and anti-myogenin (green) antibody. Shown in insets are high-magnification views. (B) Tumor masses were subjected to double immunofluorescence using an anti-myogenin (red) and anti-PAX7 (green) antibody. Notice that in TE671/WT tumors nearly all cells express PAX7 and only a few of them express myogenin, with myogenin co-localizing with PAX7 in most but not all cells where the two proteins are expressed. By contrast, in TE671/RAGE tumors the vast majority of cells are myogenin+ and only a minority of cells are PAX7+, and the two proteins are mutually exclusive. (C) Tumor masses were subjected to double immunofluorescence using an anti-Ki67 (red) and anti-myogenin (green) antibody. (Bars in A–C = 50 µm). Notice that in TE671/WT tumors essentially all cells express the proliferation marker, Ki67, whereas an extremely small fraction express myogenin. By contrast, in TE671/RAGE tumors only about half of the cells are Ki67+, a larger fraction of cells are myogenin+, and the two factors localize to different cells.
Fig. S3. Immunofluorescence analysis of PAX7, RAGE and myogenin in TE671, RD, RD12 and RD18 and TE671/RAGE ERMS cells cultivated in GM and DM. (A) TE671 cells express PAX7 but not myogenin whereas all RD, RD12 and RD18 cells express PAX7 and a fraction of them also express myogenin. By contrast, only about half of TE671/RAGE cells express PAX7, and about 20% of these cells express myogenin, but the two transcription factors are mutually exclusive. (B) TE671 cells do not express RAGE or myogenin, whereas all other tested cells types express RAGE, with a minor fraction of them also expressing myogenin. Notice that TE671/RAGE cells strongly positive for RAGE also show a strong myogenin immunofluorescence signal (arrows). (C) Whereas TE671 cells express PAX7 but not RAGE, all RD, RD12 and RD18 cells express PAX7 and a fraction of them also express RAGE. By contrast, only a minor fraction of TE671/RAGE cells express PAX7. Notice that TE671/RAGE cells strongly positive for RAGE do not show PAX7 immunofluorescence signal (arrows). (D) TE671, TE671/RAGE, RD, RD12 and RD18 ERMS cells were cultivated for 48 hours in DM and subjected to double immunofluorescence using an anti-PAX7 (green) and anti-myogenin (red) antibody. In this condition, a fraction (~30%) of TE671 cells show expression of myogenin that co-localizes with PAX7, and a larger proportion of RD, RD12 and RD18 cells exhibit expression of myogenin compared to the GM condition. Notice in TE671/RAGE cells the reduction of the number of PAX7+ cells in coincidence with an increase in the number of myogenin+ cells, and the mutually exclusive localization of PAX7 and myogenin. (Bars in A-D = 50 µm).
Fig. S4. Transient transfection of TE671/WT cells with high AGER or Myog results in RAGE and myogenin expression levels, respectively, similar to those detected in C2C12 myoblasts in DM. (A) TE671/WT cells were transiently transfected with 9 µg empty vector or 9 µg pcDNA3/human RAGE in GM. In parallel, C2C12 myoblasts were cultivated for 48 h in DM. The cells were lysed and cell lysates were subjected to Western blotting using anti-RAGE antibody. AGER-transfected TE671 cells in GM express similar levels of RAGE to C2C12 myoblasts in DM if one considers a ~30% transfection efficiency. (B) TE671/WT cells were transiently transfected in GM with increasing doses of pEMSV-mouse myogenin. In parallel, C2C12 myoblasts were cultivated for 48 h in DM. The cells were lysed and cell lysates were subjected to Western blotting using anti-myogenin antibody. TE671 cells transfected with the highest Myog dose in GM express similar levels of myogenin to C2C12 myoblasts in DM if one considers a ~40% transfection efficiency. Shown is one representative experiment of three.
Fig. S5. Effects of transient transfection of TE671 ERMS cells with either Myog or AGER. (A-D) TE671 cells were transfected in GM with increasing doses of Myog and subjected to double immunofluorescence analysis using an anti-PAX7 (green) and anti-Ki67 (red) antibody (A), an anti-myogenin (green) and anti-PAX7 (red) antibody (B), an anti-myogenin (red) and anti-MyoD (red) antibody (C), and an anti-myogenin (green) and anti-Ki67 (red) antibody (D). Percentages of positive cells are indicated. (E,F) Conditions were as in A-D except that TE671 and RD cells were transfected with increasing doses of Myog (E) or AGER (F) and cells were analyzed for apoptosis. (Bars in A-D = 50 µm). *, significantly different from control (n=3).
Fig. S6. ERMS cells express higher amounts of PAX7 than do TE671/RAGE and hu-SCs. TE671/WT, TE671/RAGE and RD cells, and hu-SCs isolated from Vastus lateralis muscles were analyzed for PAX7 expression. Ectopic expression of RAGE in TE671 cells results in reduced PAX7 expression levels.
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| Myog        | NM_031189.2      | Fwd: CAGGAGGAGGCAGATCTCCGCTAC  
                      |                      | Rev: CAGAAGTGATGGCTTTTACAACCA | 319           | 56           |
| MyoD1       | NM_010866.2      | Fwd: ATGGAGCTTCTATCGCCGACACTC  
                      |                      | Rev: GCGACCCCGCTGTGGTGGCCCTG | 276           | 60           |
| Ager        | NM_007425        | Fwd: CACTTGATGATAGCTGAAGGG  
                      |                      | Rev: CATCGCAAAATTGGATGGCTG | 180           | 60           |
| Gapdh       | NM_008084.2      | Fwd: GCCTTCGTGTTCTCTACCC  
                      |                      | Rev: CAGTGCCCTACAGATGC | 117           | 60           |
| PAX7        | NM_011039.2      | Fwd: GCAAGGCAGCGACAGCTCG  
                      |                      | Rev: GAGAAATCAGCGATGGTGGCTG | 571           | 65           |
| MYOG        | NM_002479.5      | Fwd: AACTACCTGCTGTCCACC  
                      |                      | Rev: GAGCAGGCTTCTCTACCA | 271           | 60           |
| MYOD1       | NM_002478.4      | Fwd: CGGTCCTAAAATGTAGCAGGT  
                      |                      | Rev: TCCCTGTAGCACCACACCAC | 175           | 60           |
| AGER        | NM_001136        | Fwd: AGGAGGAAGAGAGGAGGGAGGCT  
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| MyoD1| NM_010866.2      | Fwd: ATGGAGCTTCTATCGCCGCCACTC  
                     Rev: GCGACCCGCTGTTGTTGCGCCGCT | 276               |
| PAX7 | NM_002584.2      | Fwd: ACTCCACATCCAGCCCT  
                     Rev: GCGTGCTCAGAATCAAGTTC | 147               |
| MYOG | NM_002479.5      | Fwd: AAAACTACCTGCCTGTCCACC  
                     Rev: GAGCAGGGTGCTTCTTCTTCA | 271               |
| MYOD1| NM_002478.4      | Fwd: CGGTCCCAAATGTAGCAGGT  
                     Rev: TTCCCTGTAGCACACACAC | 175               |
| AGER | NM_001136        | Fwd: AGGAGGAAGAGGAGGAGCGT  
                     Rev: TGGCAAGGGTTGATACAG | 196               |
| ACTB | NM_001101.3      | Fwd: TCACCCACACTGTGCCCATCTACGA  
                     Rev: CAGCGGAACCGCTCATTTGCAATGG | 295               |