ERK signaling controls blastema cell differentiation during planarian regeneration

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SUMMARY
The robust regenerative ability of planarians depends on a population of somatic stem cells called neoblasts, which are the only mitotic cells in adults and are responsible for blastema formation after amputation. The molecular mechanism underlying neoblast differentiation associated with blastema formation remains unknown. Here, using the planarian Dugesia japonica we found that DjmkpA, a planarian mitogen-activated protein kinase (MAPK) phosphatase-related gene, was specifically expressed in blastema cells in response to increased extracellular signal-related kinase (ERK) activity. Pharmacological and genetic (RNA interference (RNAi)) approaches provided evidence that ERK activity was required for blastema cells to exit the proliferative state and undergo differentiation. By contrast, DjmkpA RNAi induced an increased level of ERK activity and rescued the differentiation defect of blastema cells caused by pharmacological reduction of ERK activity. These observations suggest that ERK signaling plays an instructive role in the cell fate decisions of blastema cells regarding whether to differentiate or not, by inducing DjmkpA as a negative regulator of ERK signaling during planarian regeneration.

KEY WORDS: Extracellular signal-related kinase (ERK), Blastema, Stem cells, Regeneration, Planarian

INTRODUCTION
Regeneration is the complex process by which animals properly reconstitute missing body parts after injury. The ability to regenerate varies greatly among animals, and is often higher in basal metazoans and lower in mammals. Key events of regeneration include successive phases of wound healing, the formation of a blastema (a mass of morphologically undifferentiated cells at the end of the stump) and differentiation to reconstitute lost tissues and organs. The origin and cellular state of blastema cells remains open to debate in many regeneration contexts (Stoick-Cooper et al., 2007) and a deeper understanding of the mechanisms underlying blastema formation and blastema cell differentiation is required to provide a basis for inducing regenerative responses in humans as novel clinical treatments for injury.

In the past decade, great advances in planarian studies have revealed many of the molecular mechanisms that regulate regeneration. The robust regenerative ability of planarians depends on a population of pluripotent stem cells called neoblasts (Baguñá, 1981; Baguñá et al., 1989; Agata and Watanabe, 1999; Newmark and Sánchez Alvarado, 2000). Neoblasts can self-renew and give rise to all missing cell types, including germ cells (Newmark et al., 2008). Neoblasts are defined morphologically as a unique population of cells (Coward, 1974; Hay and Coward, 1975; Hori, 1982; Hayashi et al., 2006; Higuchi et al., 2007) and express a specific set of ‘stemness’ genes (Shibata et al., 1999; Reddien et al., 2005; Guo et al., 2006; Oviedo and Levin, 2007; Rossi et al., 2007; Yoshida-Kashikawa et al., 2007; Eisenhoffer et al., 2008; Rouhana et al., 2010). X- or gamma-ray irradiation, which specifically eliminates neoblasts and not differentiated cells (Shibata et al., 1999), results in complete loss of regenerative ability in planarians (Wolff and Dubois, 1948). Another cellular characteristic of neoblasts is that they are the only mitotic somatic cells in adults and can therefore be visualized with universal mitotic cell markers and by BrdU incorporation (Newmark and Sánchez Alvarado, 2000; Salvetti et al., 2000; Orii et al., 2005; Hayashi et al., 2010). After amputation, neoblasts form a blastema at the end of the stump, which develops into distal structures such as the head or tail regions depending on the body axes (Agata and Watanabe, 1999; Agata et al., 2003; Agata and Umesono, 2008; Umesono and Agata, 2009). It has been reported that regeneration proceeds through two mitotic phases (Wenemoser and Reddien, 2010). The first phase occurs in response to wounding and the second occurs in response to loss of tissues after amputation. Active neoblast proliferation associated with blastema formation is found at the pre-existing tissue positioned most proximal to the blastema, termed the ‘postblastaema’ region (Sálo and Baguñá, 1984), whereas no mitosis is found within the blastema itself despite the steadily increasing number of blastema cells during regeneration (Sálo and Baguñá, 1984; Eisenhoffer et al., 2008; Wenemoser and Reddien, 2010).

Much knowledge has been gained about the functional role of many genes involved in the regulation of neoblast maintenance (for a review, see Shibata et al., 2010) by using RNA interference (RNAi) (Sánchez Alvarado and Newmark, 1999). However, the molecular mechanism underlying the switch from neoblast proliferation to differentiation associated with blastema formation remains unknown. In order to gain insight into the molecular mechanisms that regulate differentiation of neoblasts during blastema formation, we performed...
High-coverage expression profiling (HiCEP), a cDNA-amplified fragment length polymorphism (AFLP)-based gene expression profiling method (Fukumura et al., 2003). From this, we identified a mitogen-activated protein kinase (MAPK) phosphatase-related gene (DjmkpA), as a reliable marker for blastema cells in the planarian *Dugesia japonica*. Furthermore, we demonstrated that MAPK extracellular signal-related kinase (ERK) and DjmkpA form a negative-feedback loop in blastema cells that is required for proper neoblast differentiation. Our findings here provide the first molecular evidence delineating cellular events required for proper differentiation of blastema cells in planarians.

**MATERIALS AND METHODS**

**Animals**
A clonal strain of the planarian *Dugesia japonica* derived from the Iruma River in Gifu prefecture, Japan, which was maintained in autoclaved tap water at 22-24°C, was used in this study. Planarians 6-8 mm in length were starved for at least 1 week before experiments.

**HiCEP analysis**
Total RNA from ~60 fragments in each sample (head-regenerating tissues at 12 or 24 hours after amputation, tail-regenerating tissues at 12 or 24 hours after amputation, and non-regenerating tissues as a reference) was isolated using IsoGen (Nippon Gene). Using these five different sources of total RNAs as templates, HiCEP analysis was performed as described previously (Fukumura et al., 2003). Briefly, cDNA synthesized with oligo(dT) primer was digested with two restriction enzymes, TaqI and MseI, followed by the ligation of adapters. Selective PCR was then conducted using 256 primer sets, followed by capillary electrophoresis.

**Cloning of the DjmkpA cDNA by 5’-RACE**
To obtain the full-length DjmkpA sequence, 5’-RACE (rapid amplification of cDNA ends) was performed using a SMART TM RACE cDNA Amplification Kit (Clontech Laboratories). The PCR products were cloned into TA vector pGM-T Easy (Promega) and sequenced.

**X-ray and gamma-ray irradiation**
X-ray irradiation was performed as described by Yoshida-Kashikawa et al. (Yoshida-Kashikawa et al., 2007). Gamma-ray irradiation was performed with 15,000 cGy using a Gammacell 40 Exactor (Best Theratronics). Five days after irradiation, planarians were amputated and allowed to undergo regeneration.

**Whole-mount in situ hybridization**
Animals were treated with 2% hydrochloric acid (HCl) in 5/8 Holtfreter’s solution for 5 minutes at 4°C and fixed in 5/8 Holtfreter’s solution containing 4% paraformaldehyde and 5% methanol for a maximum of 2 hours at 4°C. Hybridization and color detection of digoxigenin (DIG)-labeled RNA probes were carried out as described previously by Umesono et al. (Umesono et al., 1997). TSA Labeling Kit No. 2 or No. 15 (Molecular Probes) or HNPP Fluorescent Detection Set (Roche) was used for detection of fluorescence (Yoshida-Kashikawa et al., 2007).

**In situ hybridization of sections**
Preparation of paraffin section samples was carried out as described by Takeda et al. (Takeda et al., 2009). Sections were deparaffinized with xylene and ethanol solutions of gradually decreasing concentration and acetylated with acetic acid anhydride. Then these samples were hybridized with a DIG-labeled *DjpiwiA* probe, as described by Umesono et al. (Umesono et al., 1997). To detect the signal, TSA Labeling Kit No. 15 (Molecular Probes) was used. Cell nuclei were labeled with Hoechst 33342 (Invitrogen).

**Treatment with chemical inhibitors**
The MAP/ERK kinase (MEK) inhibitor U0126 (Cell Signaling Technology) was dissolved in DMSO and used at a final concentration of 25 μM (unless otherwise indicated) in 0.25% DMSO solution. Amputated planarians were allowed to regenerate in tap water supplemented with each inhibitor either immediately after amputation or from 12 hours after amputation until the indicated period of regeneration for each experiment.

**Antibody generation and purification**
Peptide GL(pT)E(pY)VATR corresponding to the diphosphorylated form of the presumptive activation loop of the deduced protein encoded by the planarian *erk1/2*-related gene *DjerkA* was synthesized by Medical & Biological Laboratories (MBL). A rabbit polyclonal antibody against the diphosphorylated peptide was generated and affinity purified by MBL using a diphosphorylated *DjERKA* peptide column. Purified antibody fractions were further affinity purified by MBL using a non-phosphorylated *DjERKA* peptide column. Flow-through fractions from the peptide affinity column were pooled as anti-phosphorylated-specific antibody. The specificity of each antibody was confirmed by MBL by performing an enzyme-linked immunosorbent (ELISA) assay.

**Whole-mount immunostaining**
Planarians were treated with 2% HCl in 5/8 Holtfreter’s solution for 5 minutes at room temperature and washed three times with 5/8 Holtfreter’s solution at room temperature. They were then fixed in 5/8 Holtfreter’s solution containing 4% paraformaldehyde, 5% methanol and PhosSTOP phosphatase inhibitor (Roche) for 3 hours at 4°C. To decrease background signals, fixed planarians were treated with hybridization buffer for whole-mount in situ hybridization overnight at 55°C. They were then blocked with 10% goat serum in PBST (phosphate buffered saline containing 0.1% Triton X-100) for 1 hour at 4°C, and incubated with mouse anti-DjPiwiA (1/1000) (Yoshida-Kashikawa et al., 2007), rabbit anti-phosphorylated histone H3 (1/200; Upstate Biotechnology) or diluted rabbit anti-phosphorylated ERK (1/1000) overnight at 4°C. The samples were washed four times with TPBS for 30 minutes per wash. Signals were detected with Alexa Fluor 488- or 594-conjugated goat anti-rabbit or mouse IgG (1/500; Invitrogen) in 10% goat serum in TPBS for 3 hours at room temperature in the dark. TSA Labeling Kit No. 2 (Molecular Probes) was used for signal amplification.

**Western blotting**
Sixty fragments from blastemas at 1 or 2 days of regeneration were dissolved in sample buffer (0.01 M Tris-HCl, 2% SDS, 6% 2-mercaptoethanol, 10% glycerol) and were boiled for 5 minutes. The samples were then subjected to gel electrophoresis and the gel was subjected to western blotting. Blocking One-P (Nacalai Tesque) was used for membrane blocking. Western blotting was performed using rabbit anti-phosphorylated ERK (1/500), rabbit anti-DjPiwiA (1/1000) or mouse anti-α-tubulin monoclonal antibody DM 1A (1/5000; Sigma) as the primary antibody, and a 1/5000 dilution of each appropriate secondary antibody conjugated with horseradish peroxidase. Signal detection was performed using SuperSignal West Dura Extended Duration Substrate (Pierce).

**Brdu incorporation and detection**
Bromo-2′-deoxyuridine (Brdu; Sigma-Aldrich) labeling was performed by injection as described previously (Newmark and Sánchez Alvarado, 2000). Five days after injection, samples were fixed and sectioned as for in situ hybridization of sections. Deparaffinized samples were treated with 2N HCl for 30 minutes at room temperature. After washing with PBST, samples were treated overnight at 4°C with Anti-BrdU (1/25; Becton, Dickinson). BrdU signals were detected using a TSA Labeling Kit No. 2 (Molecular Probes).

**RNA interference**
Double-stranded RNA (dsRNA) was synthesized as previously described (Sánchez Alvarado and Newmark, 1999; Cebrià et al., 2002). Control animals were injected with DEPC-treated H2O alone. One day after the second or third injection, planarians were amputated and allowed to undergo regeneration.

**Reverse transcription and quantitative RT-PCR analysis**
The reverse transcription reaction was carried out with total RNA from five intact planarians or 15 tail fragments using a QuantiTect Transcription Kit (Qiagen). RT-PCR was performed as reported previously (Yazawa et al., 2000). The PCR primers used were as follows: *DjmkpA* forward, 5′-CATCTACCATCAATGGAAAGCCAG-3′; *DjmkpA* reverse, 5′-GAAGGCATCCAGTCTTCTTTCCATATATT-3′; *DjsFRP-A* forward, 5′-
RESULTS
Identification of DjmkpA, a planarian member of the mitogen-activated protein kinase phosphatase (mkp) gene family

We performed a detailed molecular analysis of early events in the process of blastema formation of the planarian D. japonica by using high-coverage expression profiling (HiCEP) (Fukumura et al., 2003). For HiCEP analysis, cDNA was prepared from five distinct sources, namely from head-regenerating tissues 12 and 24 hours after amputation, tail-regenerating tissues 12 and 24 hours after amputation, and non-regenerating tissues (time 0) as a reference (see Fig. S1 in the supplementary material). HiCEP analyses identified a gene fragment for which the expression level in each of these regenerating tissues was about ten times higher than that in the reference (data not shown). The sequence of the fragment perfectly matched that of a planarian expressed sequence tag (EST) clone encoding for a protein highly similar to mitogen-activated kinase phosphatases (MKP) (see Fig. S2 in the supplementary material). Phylogenetic analysis indicated that this fragment was not a clear ortholog of any specific vertebrate mkp gene (data not shown). Therefore, we named this gene DjmkpA (Dugesia japonica mitogen-activated kinase phosphatase A; accession number AB576208).

Spatiotemporal expression pattern of DjmkpA during regeneration

We examined the spatiotemporal expression pattern of DjmkpA during regeneration by whole-mount in situ hybridization. Planarians were transversely dissected into three pieces: head, trunk (including pharynx) and tail, and allowed to regenerate for 7 days. We performed a detailed molecular analysis of early events in the process of blastema formation of the planarian D. japonica (Ogawa et al., 2002) was used as an experimental scheme in Fig. 2P). It itself by performing a simple assay for wounding (see the detailed experimental scheme in Fig. 2P).

When the expression profiles of DjmkpA were compared between the normal and X-ray-irradiated animals, we found that expression of DjmkpA detected in differentiated cells 3 hours after amputation became apparent in X-ray-sensitive neoblast-derived blastema cells within 24 hours of amputation (Fig. 2A-E’). Thus, DjmkpA is a new reliable marker gene to monitor the processes of blastema formation during regeneration (Fig. 2Q).

DjmkpA functions as a negative-feedback regulator of ERK signaling in blastema cells

MAPK signaling pathways are evolutionarily conserved kinase cascades that regulate diverse cellular functions, including cell proliferation, differentiation, migration and response to stress (Nishida and Gotoh, 1993; Chang and Karin, 2001). In these networks, MKPs dephosphorylate active MAPK and play an important role in regulating MAPK activities (Theodosiou and Ashworth, 2002; Kondoh and Nishida, 2007). Based on the expression of DjmkpA in blastema cells, and its relationship to selectively deprived of neoblasts (Fig. 2A’-E’). During regeneration of trunk fragments, strong expression of DjmkpA was detected in the stump of the amputated regions within 3 hours of regeneration (Fig. 2B) and before the onset of DjnlgA expression at 6 hours of regeneration (Fig. 2H). DjmkpA expression at this time point was not affected by X-ray irradiation (Fig. 2B’), indicating that it originated from differentiated cells. We confirmed that the DjmkpA expression occurred in response to the wounding itself by performing a simple assay for wounding (see the detailed experimental scheme in Fig. 2P).

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MAPK signaling, we examined the activation of the MAPKs ERK and c-Jun N-terminal kinase (JNK) early in the process of blastema formation by using an antibody against phosphorylated ERK (pERK, an indicator of ERK activity; Fig. 3A) and an antibody against phosphorylated JNK (pJNK, an indicator of JNK activity). We found that activated ERK was readily detected in the blastema, and confirmed the co-expression of pERK and DjmkpA in blastema cells at the single cell level (Fig. 3A). By contrast, pJNK expression was observed not in the blastema, but in the 'postblastema' region (Tasaki et al., 2011).

To analyze the functional relationship between pERK and DjmkpA in the blastema cells, we examined the effects of various concentrations (maximum 25 μM) of a MEK inhibitor (U0126), which causes ERK inactivation (Fig. 3B), and of knockdown of the function of DjmkpA by RNAi of ERK signaling. We demonstrated by western blotting that pERK signal was already detected around the stump at time 0 (Fig. 3C). This signal was not sensitive to specific elimination of neoblasts by X-ray irradiation (Fig. 3C), indicating that ERK activation occurred in already existing differentiated cells. As regeneration proceeded, the majority of the pERK signal shifted from the differentiated cells to X-ray-sensitive neoblast-derived cells within 24 hours after amputation (Fig. 3C). Treatment with 10 μM U0126 markedly reduced the level of pERK signal derived from neoblasts (Fig. 3D), leaving the expression of phospho-histone H3 (pH3) roughly normal (Fig. 3D), demonstrating that U0126 did not affect phosphorylation levels of this different branch of the signaling pathway. We also found that treatment with U0126 reduced the DjmkpA expression in the blastema at 24 hours post-amputation in a dose-dependent manner (Fig. 3E). However, knockdown of the function of DjmkpA by RNAi increased the pERK level and caused robust resistance to pharmacological perturbation of the ERK phosphorylation level by 10 μM U0126 (Fig. 3F). The effect of DjmkpA RNAi on the ERK activation level in the blastema was confirmed by monitoring the expression level of DjsFRP-A, an ortholog of Smed-sFRP-1, which is specifically expressed in subsets of the anterior blastema-forming cells (Gurley et al., 2008; Petersen and Reddien, 2008). The expression dynamics of DjsFRP-A were highly correlated with alteration of the ERK activity levels by DjmkpA RNAi (Fig. 3G), demonstrating that DjmkpA tightly regulates the ERK downstream readout in the blastema through
the modulation of ERK signaling. Therefore, we concluded that DjmkpA functions as a negative-feedback regulator of ERK signaling in blastema cells (Fig. 3B).

**ERK inhibition caused differentiation defects of neoblasts during regeneration**

Our successful inactivation of ERK signaling by treatment of planarians with U0126 provides a method to examine the role of ERK signaling in blastema cells. Accordingly, we performed detailed histochemical analysis of the effect of ERK inhibition on temporally distinct phases of wound healing and blastema formation by assessing the expression profile of DjmkpA (Fig. 2 and Fig. 3A, A'). Planarians were allowed to regenerate in tap water supplemented with various concentrations of U0126 immediately after amputation, or 12 hours after amputation (that is, from the beginning of blastema formation after completion of wound healing) (Fig. 4A). We obtained the same results under these two conditions.

At 3 days post-amputation, we found that treatment with U0126 caused severe defects of both head and tail regeneration in a dose-dependent manner (Fig. 4B,C and Fig. 5B,D,F), although wound healing itself and the early expression of DjmkpA in differentiated cells were normal (see Fig. S3 in the supplementary material). We confirmed that this defect was also produced by U0126 treatment from 12 hours of regeneration (Fig. 4C). By contrast, as a negative control, treatment with 25 μM SB203580, a MAPK p38-specific inhibitor, resulted in normal regeneration (Fig. 4E). The regeneration defects caused by U0126 were similar to that caused by loss of neoblasts as a result of X-ray irradiation (Fig. 4C,D). However, we detected a large number of neoblasts in U0126-treated animals (Fig. 4C', D'), as shown by anti-DjPiwiA antibody staining (Yoshida-Kashikawa et al., 2007). These observations suggest that ERK signaling regulates neoblast dynamics involved in blastema formation, and not neoblast survival itself.
The planarian erk1/2-related gene DjerkA encodes an evolutionarily conserved amino acid sequence motif for phosphorylation by MEK kinase and was expressed in blastema cells at 24 hours post-amputation (see Fig. S4 in the supplementary material). We found that DjerkA(RNAi) animals phenocopied the U0126-treated animals (Fig. 5A-E). However, the majority of the DjerkA(RNAi) animals showed a head structure reduced in size and cyclopia similar to that induced by treatment with a moderate concentration (10 μM) of U0126 (Fig. 5D-F), presumably due to incomplete penetrance of DjerkA RNAi (Fig. 5G) as assayed by quantitative RT-PCR. This idea was supported by the fact that DjerkA(RNAi) animals showed phenocopy of the severe defect of 25 μM U0126-treated animals (1/30). (D) 10 μM U0126-treated animals showed cyclopia (14/30). (E) DjerkA(RNAi) animals also showed cyclopia (17/30). (F) Quantification of the phenotype in A-E based on the eye number. (G) RT-PCR analysis. DjerkA RNAi led to severe reduction of DjerkA transcripts but not of the transcripts of neoblast-specific marker genes DjpiwiA, Djpcna, DjMCM2 and DjMCM3 in intact animals. Brackets in A-E indicate the regenerating head. Scale bar: 500 μm.

ERK inhibition caused blastema cells to retain undifferentiated neoblast state

We analyzed further the regeneration defect early in the process of the blastema formation. At 24 hours post-amputation, pERK(+) cells can be recognized as a mass of non-proliferative blastema cells, in which DjPiwiA protein is strongly detected, but in which DjpiwiA mRNA expression has decayed, indicative of their undergoing a transition from the undifferentiated state to a differentiated state (Fig. 6A-C and see Fig. S6A-C in the supplementary material). Consistent with the normal survival of neoblasts, accumulation of DjPiwiA(+) cells at both the anterior and posterior stumps (presumptive blastema cells) was readily observed in U0126-treated regenerates at 24 hours post-amputation (Fig. 6D,E and see Fig. S7 in the supplementary material). However, these cells sustained high levels of phosphorylation by MEK kinase and was expressed in blastema cells at 24 hours post-amputation and posterior stumps (presumptive blastema cells) was readily observed in U0126-treated regenerates at 24 hours post-amputation and posterior stumps (presumptive blastema cells) was readily observed in U0126-treated regenerates at 24 hours post-amputation.

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ERK is required for the differentiation of neoblasts into multiple cell lineages

The normal physiological cell turnover in non-regenerating intact planarians depends on neoblasts (Pellettiere and Sánchez Alvarado, 2007), and thus provides another opportunity to examine the effect of ERK inhibition on neoblast differentiation without injury to the animals. To trace neoblast progeny at the single cell level, neoblasts were specifically labeled by BrdU incorporation (Newmark and...
Sánchez Alvarado, 2000), and allowed to undergo cell differentiation for 5 days in water (as control) or water supplemented with 25 μM U0126. In intact animals, brain, gut and pharynx structures were visualized by Hoechst staining (Fig. 7Ai-iii). The lack of DjpiwiA expression in these organs indicates that they are composed solely of differentiated cells (Fig. 7A). In control animals, the first detectable signal was observed only in DjpiwiA mRNA(+) undifferentiated neoblasts at 24 hours post-BrdU pulse (data not shown). Five days after the BrdU-pulse, BrdU incorporation was detected in DjpiwiA mRNA(–) neoblast progeny from a variety of tissues derived from the three germ layers, such as the brain, pharynx and intestinal tube (Fig. 7Ai-iii). At this time-point, we could not detect BrdU incorporation in epidermal cells. Consistent with our hypothesis, U0126-treated animals showed normal BrdU incorporation in DjpiwiA mRNA(+) undifferentiated neoblasts in the mesenchymal space 5 days after BrdU-pulse labeling (Fig. 7B,C), but a drastic decrease in the number of BrdU(+) cells in a variety of differentiated tissues, including the brain, pharynx and intestinal tube (Fig. 7Bi-iii,D). From these results, we conclude that ERK signaling is not required for survival and self-renewal of neoblasts, but is required for their differentiation into multiple cell lineages in planarians.

An ERK-DjmkpA feedback circuit makes the cell fate decision of blastema cells

To clarify whether DjmkpA indeed functions in the circuitry of the ERK-dependent cell fate decisions, we performed a functional assay to test the effect of DjmkpA RNAi on blastema cells sensitized by treatment with a concentration of U0126 that leads to inhibition of the ERK activity at a moderate level. Head regeneration from tail fragments was used as a model to assess the ability of DjmkpA RNAi to rescue these defects. U0126 (10 μM) caused clear differentiation defects in the head region, such as its reduction in size with cyclopia or no eyes, in all of the treated animals at 7 days post-amputation (Fig. 8C). This defect was robustly rescued by simultaneous DjmkpA RNAi (Fig. 8D), which accords with the restoration of the ERK phosphorylation level (Fig. 3F, lane 4). Quantitative analysis (counting the number of successfully regenerated eyes) confirmed that DjmkpA RNAi rescued the regenerative defects induced by U0126 treatment (Fig. 8E).

Based on all these findings, we conclude that negative-feedback regulation of ERK signaling by DjmkpA seems to establish a threshold level of ERK activation for binary cell fate decisions of blastema cells regarding whether to proliferate or to differentiate.

**DISCUSSION**

**Role of ERK signaling in blastema cells**

Here, we clarified the details of stem cell dynamics early in the process of blastema formation during regeneration of the planarian Dugesia japonica. On the basis of our findings, we propose that blastema cells originate from a population of proliferating neoblasts near the amputation stump (postblastema) (Sálo and Baguñà, 1984) and subsequently undergo activation of ERK signaling that allows the blastema cells to simultaneously exit from the proliferative state and enter the differentiating state. ERK-dependent switching of the
cellular state of neoblasts results in a clear distinction between the blastema (non-proliferative) and postblastema (proliferative) regions (Fig. 9A,B).

ERK signaling is also known to regulate cytoskeletal machineries that drive cell migration in many developmental contexts (Huang et al., 2004; Pullikuth and Catling, 2007), but, in fact, treatment with U0126 did not affect the migration of blastema cells towards the amputation stump in D. japonica, because U0126-treated animals showed a blastema-like structure (Fig. 6E). However, reduced directional migration of blastema cells into their correct locations within the blastema could still account for the regeneration defect induced by U0126 (Fig. 9A,B). It has been reported that neoblast migration and differentiation are strongly coupled with each other in planarians (Newmark and Sánchez Alvarado, 2000). Therefore, our observations encourage us to speculate that ERK signaling might promote blastema cell differentiation and coordinate blastema cell migration, resulting in the robust formation of the blastema structure during regeneration.

Why did ERK inhibition result in a decreased rather than increased number of blastema cells? To ensure continuous recruitment of neoblast progeny to the blastema at a later stage, ERK signaling might play a role in directing active mitosis in a non-cell autonomous manner after the formation of the blastema. Agata et al. (Agata et al., 2003; Agata et al., 2007) previously proposed that a newly formed blastema acts as a signaling center that induces intercalation to restore patterning along the anterior-posterior axis during planarian regeneration. In this scenario, we suggest that ERK signaling ensures the differentiation of an initial cohort of blastema cells early in the process of regeneration (around 24 hours post-amputation). This is required for subsequent blastema function as a distalization signal that induces active cellular state of neoblasts results in a clear distinction between the blastema (non-proliferative) and postblastema (proliferative) regions (Fig. 9A,B).

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mitosis of undifferentiated neoblasts outside the blastema to feed their progeny into a variety of regenerating tissues, including the blastema during intercalary regeneration. (We believe that mitosis in D. japonica is likely to correspond to the second mitotic phase in Schmidtea mediterranea). This idea at least would account for the most puzzling observation that ERK inhibition decreased number of blastema cells at 72 hours post-amputation (Fig. 4B’C’). We are looking forward to assessing our proposed model in the future.

Recently accumulated knowledge about regenerative programs in many model organisms highlights crucial roles of MAPK signaling pathways early in the regenerative response (Tanaka and Galliot, 2009). Importantly, ERK activation has been found in developing blastemas in various animal species (Suzuki et al., 2007; Nakamura et al., 2008). These observations suggest that ERK activation might act as a common molecular indicator of the differentiating state of blastema cells among animal species.

**DjmkpA functions as a negative-feedback regulator of the ERK signaling in blastema cells**

MKPs inactivate JNK, ERK and p38 MAPKs by dephosphorylation with distinct specificities for individual MKPs (Theodosiou and Ashworth, 2002; Kondoh and Nishida, 2007). The first wave of DjmkpA expression occurred immediately in differentiated cells in response to wounding stimuli independently of ERK signaling. By contrast, we found that DjmkpA acts as a negative-feedback regulator of ERK signaling in blastema cells (Fig. 3 and Fig. 8C). This feedback circuit would ensure pivotal cell fate decisions of blastema cells, namely proliferation or differentiation, in the presence of fluctuations generated by upstream noise and variations of ERK signaling. We speculate that the expression of DjmkpA is involved in inactivation of ERK, but not JNK, based on the spatially distinct profiles of their activation during regeneration (Tasaki et al., 2011). Our recent study demonstrated that strong JNK activation is observed in the postblasta region around 24 hours post-amputation and is required for the entry of neoblasts into M-phase of the cell cycle and for the generation of blastema cells, in which ERK is highly activated (Tasaki et al., 2011) (J.T., unpublished data). If DjmkpA were required for inactivation of JNK in the blastema under the control of ERK activity, the U0126-induced differentiation defect would be enhanced by simultaneous DjmkpA RNAi, presumably owing to disturbance of the cell fate transitions of blastema cells by JNK-dependent active mitosis. In fact, DjmkpA (RNAi) did not seem to rescue the regeneration defect induced by the treatment with the JNK inhibitor (J.T., unpublished data).

**Implications for stem cell biology**

Historically, it was thought that during vertebrate regeneration blastema cells de-differentiate into a undifferentiated pluripotent state, as typified by embryonic stem (ES) cells, which are derived from the inner cell mass of mammalian blastocysts and have the ability to self-renew and differentiate into cells of ectodermal, endodermal and mesodermal germ layers (Evans and Kaufman, 1981; Martin, 1981). However, recent work on the salamander *Ambystoma mexicanum* showed that blastema cells have apparently restricted potential to differentiate during limb regeneration (Kragl et al., 2009). Although somatic cells can be reprogrammed to a pluripotent state by the expression of defined factors in mammalian cells (Takahashi and Yamanaka, 2006), during the normal course of regeneration, blastema cells in the salamander are in a cellular state apparently different from that of ES cells.

Our findings demonstrated that the cellular state of blastema cells in planarians fits the conventional concept of blastema cells when the ERK signaling is inhibited, for they maintain their pluripotent neoblast state. This leads to the interesting idea that neoblasts in planarians and ES cells in vertebrates might share a common molecular basis for their cell fate decisions (Fig. 9C). That
is, proliferation of neoblasts is highly sensitive to ERK signaling as a differentiation signal in a similar way to the regulation of ES cells in mice (Ying et al., 2008). Similarities between planarian neoblasts and mammalian ES cells have been postulated previously by their similar expression of homologous RNA-binding proteins (Salvetti et al., 2005; Shibata et al., 2010; Rouhana et al., 2010). It is known that fibroblast growth factor (FGF) signaling acts upstream of ERK signaling to promote cell fate decisions of ES cells in mice (Kunath et al., 2007). Mkp3 (Dusp6) is a well-known target of the FGFR-mediated ERK signaling pathway in vertebrates, and encodes a key modulator that determines the magnitude and duration of ERK activity in a negative feedback manner (Eblaghie et al., 2003; Smith et al., 2006; Li et al., 2007). This suggests that Mkp3 in vertebrates and DmMkpA in planarians share a similar function in the ERK-dependent differentiation network. Furthermore, identification of the nou-darake gene provided strong molecular evidence for the existence of a brain-inducing circuit based on FGF signaling in planarians (Cebrià et al., 2002). These observations lead us to speculate that FGF signaling and an ERK/mkp feedback circuit were integrated into the pluripotent stem cell system early in the course of evolution and together play a crucial role in balancing the self-renewal and differentiation of stem cells (Lanner and Rossant, 2010). Therefore, we study highlights the possible value of planarian studies for regenerative medicine via integration of regeneration biology and stem cell biology.

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Competing interests statement
The authors declare no competing financial interests.

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Blastema cell differentiation in planarians


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