

# MT1-MMP cleaves Dll1 to negatively regulate Notch signalling to maintain normal B-cell development

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**Notch signalling controls the differentiation of haematopoietic progenitor cells (HPCs). Here, we show that loss of membrane-type 1 matrix metalloproteinase (MT1-MMP, MMP14), a cell surface protease expressed in bone marrow stromal cells (BMSCs), increases Notch signalling in HPCs and specifically impairs B-lymphocyte development. When co-cultured with BMSCs *in vitro*, HPCs differentiation towards B lymphocytes is significantly compromised on MT1-MMP-deficient BMSCs and this defect could be completely rescued by DAPT, a specific Notch signalling inhibitor. The defective B-lymphocyte development could also be largely rescued by DAPT *in vivo*. MT1-MMP interacts with Notch ligand Delta-like 1 (Dll1) and promotes its cleavage on cell surface in BMSCs. Ectopic MT1-MMP cleaves Dll1 and results in diminished Notch signalling in co-cultured cells. In addition, recombinant MT1-MMP cleaves a synthetic Dll1 peptide at the same site where MT1-MMP cleaves Dll1 on the cell surface. Our data suggest that MT1-MMP directly cleaves Dll1 on BMSCs to negatively regulate Notch signalling to specifically maintain normal B-cell development in bone marrow.**

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## Introduction

Notch signalling has an important role in regulating intercellular communication, which is essential for various

biological functions, such as cell fate decision, proliferation, survival and apoptosis (Artavanis-Tsakonas *et al.*, 1999; Bray, 2006). It is involved in diverse developmental processes of organs, tissues and cells (Conlon *et al.*, 1995; Radtke *et al.*, 1999; Gaiano *et al.*, 2000; Huppert *et al.*, 2000; Krebs *et al.*, 2000; Morrison *et al.*, 2000; Morrow *et al.*, 2008). In mammalian cells, the Notch signalling pathway is composed of four receptors (Notch1, 2, 3 and 4) and five ligands (Jagged1 and 2, and Delta-like 1 (Dll1), 3 and 4), which are all expressed on the cell surface. The binding of ligands to Notch receptors transmits Notch signalling to neighbouring cells. The interaction first triggers a stepwise processing of Notch receptor and releases the intracellular domain termed Notch intracellular domain (NICD; Schroeter *et al.*, 1998; Brou *et al.*, 2000; Mumm *et al.*, 2000). Then, NICD translocates into the nucleus and forms a complex with CSL DNA binding protein (also known as CBF1 and RBP-J $\kappa$ ) and other co-activators to transactivate downstream target genes such as *Hes1* and *Hes5* (Jarriault *et al.*, 1995; Wu *et al.*, 2000).

The contribution of Notch signalling to early lymphocyte development has been well described. Notch signalling favours T-cell commitment from common progenitor cells at the expense of B-cell development. This has been supported by various loss or gain of function analyses of Notch pathway components. For example, Notch1 deletion leads to a block of thymus T-cell development but promotes abnormal B-cell commitment (Radtke *et al.*, 1999; Wilson *et al.*, 2001). Similarly, the deletion of RBP-J $\kappa$  leads to the same defect of early lymphocyte development (Han *et al.*, 2002). In addition, overexpression of activated Notch1 (aNotch1) in haematopoietic progenitor cells (HPCs) results in reduction in B-cell population in bone marrow but promotion of T-cell commitment (Pui *et al.*, 1999). Overexpression of Notch signalling target genes, the transcription factors *Hes5* and *Hes1*, partially block B-cell development (Kawamata *et al.*, 2002; Hoebeke *et al.*, 2006). Furthermore, deletion of the Notch negative regulator LRF also blocks B-cell development and promotes T-cell commitment (Maeda *et al.*, 2007). The critical role of Notch signalling in lymphocyte development is further supported by a well-established *in vitro* co-culture system. Stromal cells overexpressing Notch ligand Dll1 initiate the T-cell commitment at the expense of B-cell development from co-cultured HPCs (Jaleco *et al.*, 2001; Schmitt and Zuniga-Pflucker, 2002).

In mammalian bone marrow, fibroblast-like stromal cells contribute critically to the development of haematopoietic cells, including stem cell maintenance and lineage cell development, by direct contact or release of cytokines (Nagasawa, 2006). Notch ligands, including Dll1, are expressed by the stromal cells whereas Notch receptors are widely expressed on haematopoietic cells (Radtke *et al.*, 2004), suggesting that Notch signalling is involved in the haematopoietic system. However, in bone marrow, only B lymphocytes and not T cells

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are generated. These studies indicate that Notch signalling might be negatively regulated to maintain normal B-cell development. The cleavage of Dll1 from the cell surface is one potential way to inactivate Dll1-induced Notch signalling. It has been shown that Dll1 is cleaved by various proteases of the ADAM (A Disintegrin And Metalloproteinase) family (Six *et al*, 2003; Dyczynska *et al*, 2007). The cleavage of Dll1 by ADAM10 impairs Notch signalling and consequently causes defective self-renewal in neural precursor cells (Muraguchi *et al*, 2007).

MT1-MMP, encoded by *MMP14*, is an important cell surface metalloproteinase that plays critical roles in matrix degradation and regulation of receptor signalling. MT1-MMP is expressed predominantly in bone tissues including bone marrow. Deficiency in MT1-MMP significantly impairs the endochondral ossification. However, it is not known if loss of MT1-MMP affects lymphocyte development. Here, we show loss of MT1-MMP enhances Notch signalling in HPCs and partially blocks B-cell development in bone marrow. Purified bone marrow stromal cells (BMSCs) from MT1-MMP-deficient mice enhance Notch signalling and inhibit B-cell commitment in co-cultured HPCs. The defective B-cell development could be rescued *in vitro* and *in vivo* by a specific Notch signalling inhibitor. MT1-MMP in BMSCs facilitates the cleavage of Notch ligand Dll1. In addition, recombinant MT1-MMP cleaves Dll1 peptide at a novel site different from that cleaved by ADAMs. These data suggest that MT1-MMP directly cleaves Dll1 and negatively regulates Dll1-induced Notch signalling in HPCs, thus specifically modulating B-lymphocyte differentiation in bone marrow.

## Results

### Abnormal B-cell development in MT1-MMP-deficient bone marrow

To investigate whether MT1-MMP is required for early development of lymphocytes, we collected total bone marrow from wild-type and MT1-MMP-deficient mice aged day 10–14 to analyse various lymphocyte populations. The percentage of total B cells, including early stage (B220<sup>+</sup>CD43<sup>+</sup>) and late stage (B220<sup>+</sup>CD43<sup>-</sup>), was both dramatically declined in MT1-MMP-deficient mice (Figure 1A and C). The pre-pro B cells identified as the B220<sup>+</sup>CD43<sup>+</sup>CD24<sup>low/-</sup> population and the pro B cells identified as the B220<sup>+</sup>CD43<sup>+</sup>CD24<sup>high</sup> population (Nagasawa, 2006) were found to be significantly reduced in MT1-MMP-deficient bone marrow compared with that in wild types (Figure 1A–C). Consistently, the B220<sup>+</sup>CD19<sup>-</sup>AA4.1<sup>+</sup> early stage B cells and B220<sup>+</sup>CD19<sup>+</sup>AA4.1<sup>+</sup>, B220<sup>+</sup>CD19<sup>+</sup>AA4.1<sup>-</sup> late stage B cells were also reduced in bone marrow from MT1-MMP-deficient mice (Supplementary Figure S1). However, both B220<sup>+</sup>CD19<sup>-</sup>AA4.1<sup>+</sup> early stage B cells and B220<sup>+</sup>CD19<sup>+</sup>AA4.1<sup>+</sup>, B220<sup>+</sup>CD19<sup>+</sup>AA4.1<sup>-</sup> late stage B cells in the fetal liver of MT1-MMP-deficient mice were comparable to that in wild types (Supplementary Figure S2), suggesting that no significant impairment in B-cell development was observed in MT1-MMP-deficient fetal liver. These data indicate that the B-cell development defect occurs at relatively early stage specifically in the bone marrow of MT1-MMP-deficient mice, suggesting that MT1-MMP is required for the commitment of bone marrow haematopoietic progenitors to the B-cell lineage.

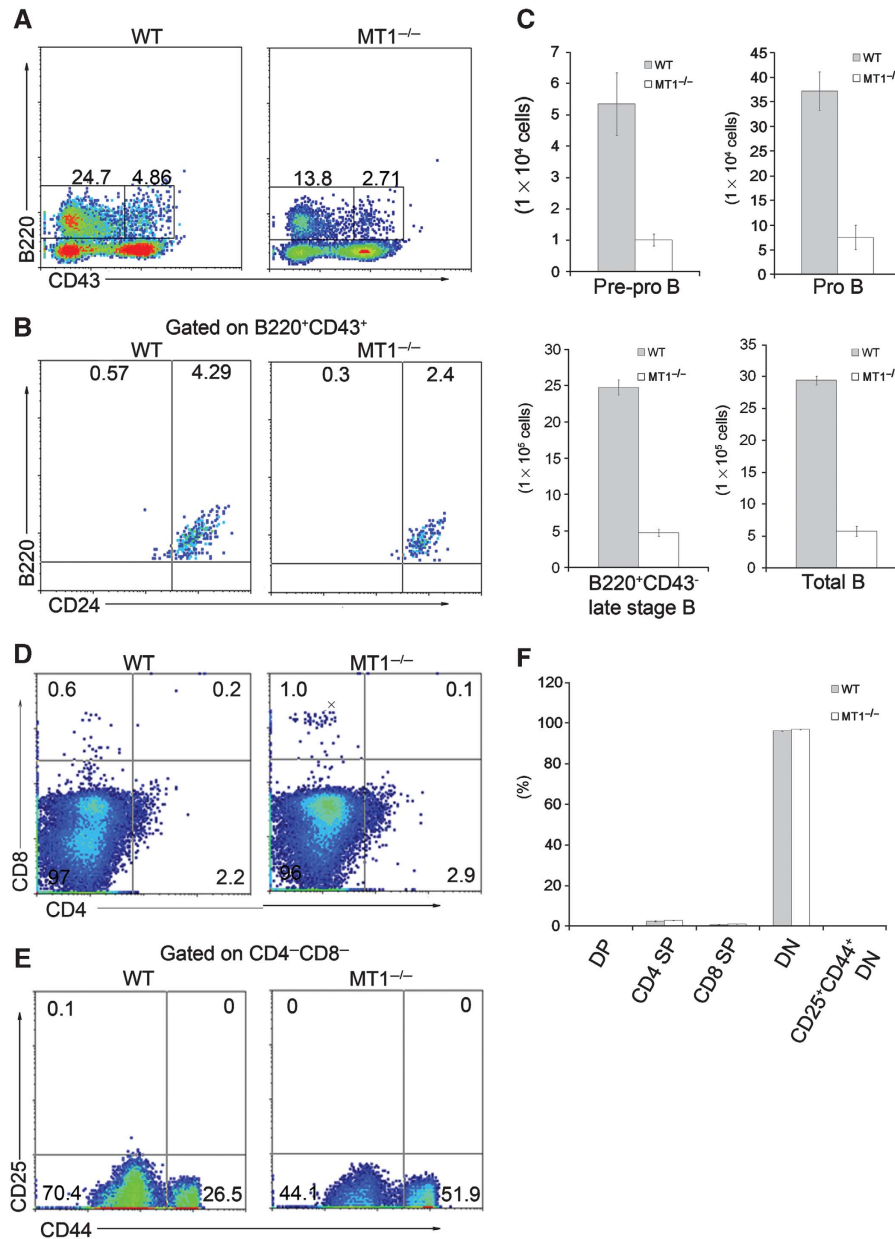
We then examined the T-cell populations in bone marrow. There were small populations of CD4<sup>+</sup> single positive T cells (CD4 SP) and CD8<sup>+</sup> single positive T cells (CD8 SP) in either wild-type or MT1-MMP-deficient bone marrow (Figure 1D and F). These cells were recruited from the peripheral circulation, because the CD4<sup>+</sup>CD8<sup>+</sup> double positive T cells (DP) and immature CD4<sup>-</sup>CD8<sup>-</sup>CD25<sup>+</sup>CD44<sup>+</sup> T-cell progenitors (CD25<sup>+</sup>CD44<sup>+</sup>DN) were hardly detectable in bone marrow of either wild-type or MT1-MMP-deficient mice (Figure 1D–F). The T-cell populations in thymus were also examined. No significant difference was observed in either CD4 SP, CD8 SP and CD4CD8 DP T-cell populations (Supplementary Figure S3A and B), or DN1 (CD4<sup>-</sup>CD8<sup>-</sup>CD25<sup>-</sup>CD44<sup>+</sup>), DN2 (CD4<sup>-</sup>CD8<sup>-</sup>CD25<sup>+</sup>CD44<sup>+</sup>), DN3 (CD4<sup>-</sup>CD8<sup>-</sup>CD25<sup>+</sup>CD44<sup>-</sup>) and DN4 (CD4<sup>-</sup>CD8<sup>-</sup>CD25<sup>-</sup>CD44<sup>-</sup>) progenitors (Supplementary Figure S3C and D), between wild-type and MT1-MMP-deficient mice. These data suggest that T-cell commitment in either bone marrow or thymus is not significantly affected in MT1-MMP-deficient mice.

### Abnormal B-cell development in MT1-MMP-deficient mice may not be cell autonomous

To understand whether the abnormal B-cell development in MT1-MMP-deficient mice is cell autonomous, bone marrow reconstitution experiments were performed. Total bone marrow from either wild-type mice or MT1-MMP-deficient mice (both of CD45.2 background) was transplanted into lethally irradiated adult wild-type mice of CD45.1 background. Six months later, total bone marrow cells were collected and B-cell populations were analysed. High reconstitution efficiencies were achieved, as demonstrated by the ratio of CD45.2<sup>+</sup> cells to total CD45<sup>+</sup> cells (Supplementary Figure S4). In the reconstituted adult mice, two B220<sup>+</sup> populations (B220<sup>high</sup> and B220<sup>low</sup>) were observed. It has been shown that the B220<sup>high</sup> population represents the recruited mature B cells. The B220<sup>low</sup> population represents the bone marrow-derived lineage B-cell precursors (Masuzawa *et al*, 1994). We use the ratio of CD45.2<sup>+</sup>B220<sup>low</sup> cells to CD45.2<sup>+</sup>(CD45.2<sup>+</sup>B220<sup>low</sup> plus CD45.2<sup>-</sup>B220<sup>-</sup> population) cells to measure the differentiation capacity of B cells from donor HPCs. The B-cell differentiation in bone marrow was comparable between recipients reconstituted with MT1-MMP-deficient bone marrow and recipients reconstituted with wild-type bone marrow (Figure 2A and B). There was no significant difference in CD3<sup>+</sup> T-cell populations between wild-type and MT1-MMP-deficient bone marrow recipients after bone marrow reconstitution (Figure 2C and D). These data indicate that the abnormal B-cell development in MT1-MMP-deficient mice is likely not resulted from intrinsic defects in haematopoietic cells, but rather a consequence of the defective niche, such as the BMSCs.

### BMSCs express MT1-MMP

MT1-MMP has been shown to be expressed in mesenchymal derived osteoblast cells and adipocytes (Kinoh *et al*, 1996; Apte *et al*, 1997; Chun *et al*, 2006). To understand why loss of MT1-MMP results in defective B-cell development, we first examined the expression of MT1-MMP in bone marrow. Paraffin sections of mouse long bones were prepared and MT1-MMP expression was assessed by immunohistochemistry. The fibroblast-like cells known as bone marrow mesenchymal stromal cells were positive for MT1-MMP. The small

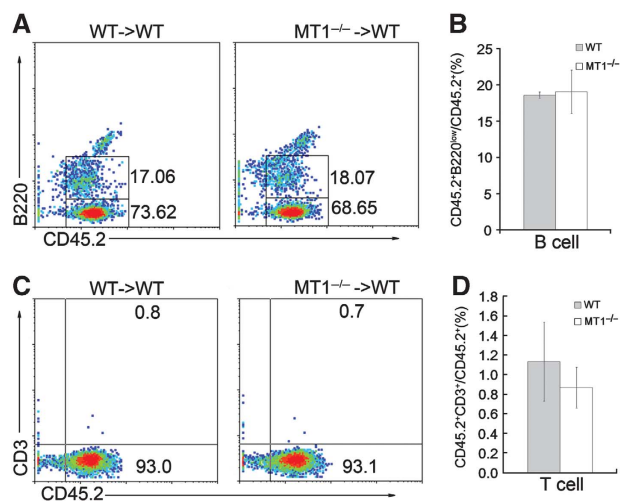


**Figure 1** MT1-MMP deficiency impairs B-cell development in bone marrow. (A) Flow cytometry analyses of early stage and late stage B-cell populations in either wild-type (WT) or MT1-MMP-deficient (MT1<sup>-/-</sup>) bone marrow. Numbers adjacent to the outlined areas indicate the percentage of early stage B220<sup>+</sup>CD43<sup>+</sup> cells (right) or late stage B220<sup>+</sup>CD43<sup>-</sup> cells (left). (B) Flow cytometry analyses of B cells gated on B220<sup>+</sup>CD43<sup>+</sup> population by the expression of CD24. Numbers in the plots indicate the percentage of B220<sup>+</sup>CD43<sup>+</sup>CD24<sup>low/-</sup> cells (left) or B220<sup>+</sup>CD43<sup>+</sup>CD24<sup>high</sup> cells (right). (C) Statistical analyses of different stages of B-cell development in bone marrow;  $P=0.0004$  (pre-pro B),  $P=0.0004$  (pro B),  $P<0.0001$  (B220<sup>+</sup>CD43<sup>-</sup> B),  $P<0.0001$  (total B), Student's *t*-test. Data are representative of three independent experiments (mean  $\pm$  s.d.). (D) Flow cytometry analyses of bone marrow T cells. Numbers in the plots indicate the percentage of CD4 SP (bottom-right), CD8 SP (up-left), DP (up-right) and DN (bottom-left) cells. (E) Flow cytometry analyses of bone marrow CD44<sup>+</sup>CD25<sup>+</sup> DN T-cell progenitors. Numbers in the plots indicate the percentage of CD44<sup>+</sup>CD25<sup>+</sup> DN cells (up-right). (F) Statistical analyses of T-cell populations in bone marrow;  $P>0.05$ , Student's *t*-test. Data are representative of two independent experiments (mean  $\pm$  s.d.). DP, CD4CD8 double positive; DN, CD4CD8 double negative; CD4 SP, CD4 single positive; CD8 SP, CD8 single positive.

haematopoietic cells in the bone marrow derived from wild-type mice were negative for MT1-MMP immunostaining. As expected, the immunostaining was negative in bone marrow from MT1-MMP-deficient mice (Figure 3A). The expression of MT1-MMP in purified BMSCs was further confirmed by western blotting. Total bone marrow cells from long bones (femurs and tibias) were isolated and cultured for 2 weeks. The adherent fibroblast-like BMSCs were collected, sorted to remove contaminated lineage-positive haematopoietic cells

and subcultured *in vitro*. The isolated BMSCs from both wild-type and MT1-MMP-deficient mice showed similar morphology in culture. MT1-MMP was highly expressed in wild-type BMSCs (Figure 3B and C). Thus, both *in vivo* and *in vitro* results show that MT1-MMP is highly expressed in mouse BMSCs.

We then examined the expression of MT1-MMP in HPCs. Lineage-negative Sca1<sup>+</sup>cKit<sup>+</sup> HPCs were sorted out from total bone marrow of wild-type mice. Western blotting



**Figure 2** Reconstitution of either wild-type or MT1-MMP-deficient bone marrow cells into lethally irradiated wild-type mice. (A) Flow cytometry analyses of bone marrow B cells 6 months after bone marrow reconstitution. Numbers in the plots indicate the percentage of CD45.2<sup>+</sup>B220<sup>low</sup> cells (up) or CD45.2<sup>+</sup>B220<sup>+</sup> cells (bottom). (B) Statistical analysis of B-cell commitment from donor bone marrow cells after bone marrow reconstitution;  $P > 0.05$ , Student's *t*-test. Data are representative of two independent experiments (mean  $\pm$  s.d.). (C) Flow cytometry analyses of bone marrow T cells 6 months after bone marrow reconstitution. Numbers in the plots indicate the percentage of CD45.2<sup>+</sup>CD3<sup>+</sup> cells (up) or CD45.2<sup>+</sup>CD3<sup>-</sup> cells (bottom). (D) Statistical analysis of T-cell commitment from donor bone marrow cells after bone marrow reconstitution;  $P > 0.05$ , Student's *t*-test. Data are representative of two independent experiments (mean  $\pm$  s.d.).

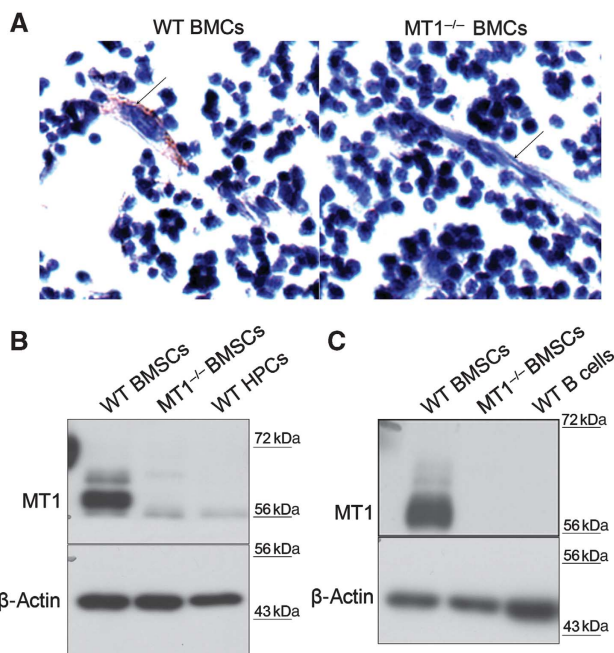
showed no detectable MT1-MMP in either sorted HPCs (Figure 3B) or B220<sup>+</sup> B lymphocytes isolated from bone marrow (Figure 3C). Therefore, MT1-MMP is expressed in BMSCs but not in haematopoietic cells—at least not in HPCs or B cells. These data are in line with the observation that the defect in B-cell development in MT1-MMP-deficient mice is not cell autonomous.

### Notch1 signalling is abnormally increased in MT1-MMP-deficient haematopoietic progenitors and committed B lineage cells

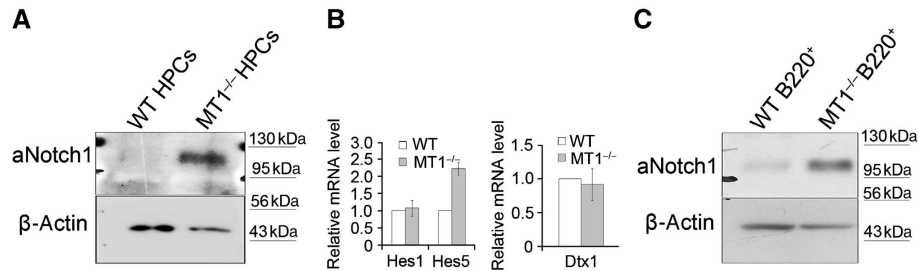
To understand the underlying mechanism of defective B-cell development in bone marrow from MT1-MMP-deficient mice, we analysed the potential signalling alterations caused by MT1-MMP deletion. Previous studies have shown that Notch signalling has a critical role in early lymphocyte commitment. Activation of Notch signalling in haematopoietic progenitors blocks B-cell development and induces abnormal T-cell commitment in bone marrow (Pui *et al*, 1999). It is not surprise that no abnormal T-cell initiation is observed in MT1-MMP-deficient bone marrow as it has been shown that a much higher level of Notch signalling is required to induce T-cell development than to block B-cell development (Schmitt *et al*, 2004). In addition, overexpression of either Hes1 or Hes5 partially blocks B-cell development without affecting T-cell maturation (Kawamata *et al*, 2002). Therefore, it is possible that abnormal Notch signalling in MT1-MMP-deficient bone marrow may be sufficient to affect B-cell development but not to the level affecting T-cell commitment. To test whether the defective B-cell development in MT1-MMP-deficient mice is a consequence of abnormal Notch signalling, the lineage-negative Sca1<sup>+</sup>cKit<sup>+</sup> HPCs were isolated from both wild-type and MT1-MMP-deficient bone marrow for Notch signalling analyses. The aNotch1 (the released intracellular domain of Notch1) signalling was significantly increased in the HPCs from MT1-MMP-deficient mice, compared with that from wild-type mice (Figure 4A). Consistently, the mRNA level of Notch target *Hes5*, which could block B-cell development (Kawamata *et al*, 2002), was significantly upregulated in MT1-MMP-deficient HPCs, though the mRNA levels of Notch target *Hes1* and Notch regulator *Dtx1* (*Deltex1*) were not much affected (Figure 4B). In addition, significant upregulation of aNotch1 was found in B220<sup>+</sup> cells in MT1-MMP-deficient mice, compared with that in wild-type mice (Figure 4C). These results indicate that loss of MT1-MMP enhances Notch1 signalling, which in turn impairs B-cell development in bone marrow.

### MT1-MMP-deficient BMSCs impairs B-cell development due to increased Notch signalling

Since B-cell development defect in MT1-MMP-deficient mice might be resulted from altered BMSCs functions (Figures 2A and B and 3), we cultured wild-type HPCs on wild-type and MT1-MMP-deficient BMSCs to examine Notch signalling in the HPCs. Significantly higher levels of aNotch1 protein and *Hes5* mRNA were found in HPCs co-cultured on MT1-MMP-deficient BMSCs (Figure 5A and B). These results support the idea that MT1-MMP in stromal cells is required to inhibit Notch signalling in HPCs. To further investigate whether the defective B-cell development in MT1-MMP-deficient mice is a consequence of enhanced BMSCs-mediated Notch signalling, an *in vitro* co-culture system was employed to examine the



**Figure 3** MT1-MMP is expressed in BMSCs but not in HPCs. (A) Immunostaining analyses of MT1-MMP in wild-type bone marrow section. MT1<sup>-/-</sup> bone marrow section serves as negative control. Arrows indicate fibroblast-like stromal cells. (B, C) Immunoblot analyses of MT1-MMP expression in BMSCs, HPCs and B cells. MT1<sup>-/-</sup> BMSCs serve as negative control.  $\beta$ -Actin serves as an internal loading control.



**Figure 4** Loss of MT1-MMP increases Notch1 signalling in either HPCs or B cells. (A) Immunoblot analyses of aNotch1 in isolated lineage-negative Sca1<sup>+</sup>cKit<sup>+</sup> HPCs. (B) Real-time PCR analyses of mRNA levels of *Hes1*, *Hes5* and *Dtx1* in isolated HPCs from wild-type and MT1-MMP<sup>-/-</sup> bone marrow;  $P=0.0011$  (*Hes5*), Student's *t*-test. Data are representative of three independent experiments (mean  $\pm$  s.d.). (C) Immunoblot analyses of aNotch1 in isolated B220<sup>+</sup> B cells.  $\beta$ -Actin serves as an internal loading control (A, C).

differentiation of HPCs. Lineage-negative Sca1<sup>+</sup>cKit<sup>+</sup> HPCs were cultured on top of the BMSCs for 7 days before the commitment into B cells was analysed. A significantly lower percentage of B-cell commitment was found from HPCs cultured on MT1-MMP-deficient BMSCs, compared with those cultured on wild-type BMSCs. When Notch signalling was inhibited by DAPT in the co-culture system, the defective B-cell development from HPCs cultured on MT1-MMP-deficient BMSCs was restored (Figure 5C and D), suggesting that defective B-cell development mediated by BMSCs in MT1-MMP-deficient bone marrow is Notch signalling dependent. However, similar to the *in vivo* data, CD3<sup>+</sup> T-cell population was not observed in the co-culture system (Figure 5C). Further examination of the T-cell commitment after 2 weeks co-culture revealed that neither late stage T cells (CD4 SP and CD8 SP), nor earlier T-cell progenitors (CD4CD8 DP and CD25<sup>+</sup>CD44<sup>+</sup> DN) were detectable (Figure 5E and F), suggesting that enhanced Notch signalling mediated by MT1-MMP-deficient BMSCs specifically impairs B-cell development, but is not sufficient to promote T-cell development (Schmitt *et al*, 2004).

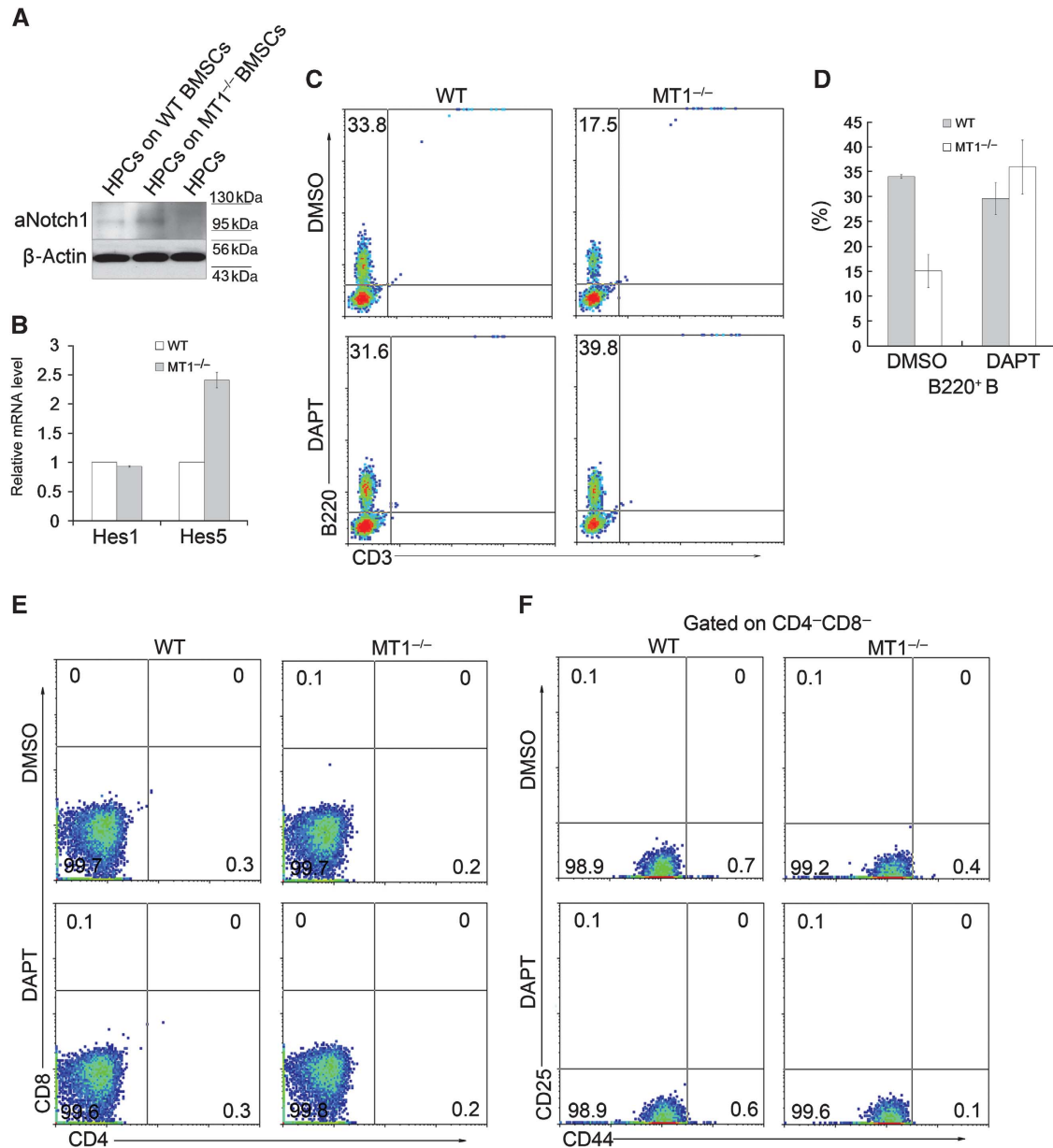
To substantiate the observation that lack of MT1-MMP in BMSCs can increase the transmission of Notch signalling, we employed a well-characterized co-culture system in which the differentiation of C2C12 myoblasts into myotubes is inhibited by Notch signalling (Shawber *et al*, 1996; Nofziger *et al*, 1999; Lehar *et al*, 2005). The C2C12 cells were co-cultured on either wild-type or MT1-MMP-deficient BMSCs to induce myotube formation *in vitro*. The  $\gamma$ -secretase inhibitor DAPT was used to inhibit Notch signalling. Significantly reduced myotube formation was observed in C2C12 cells cultured on MT1-MMP-deficient BMSCs (Figure 6A). The mRNA levels of both *Mlc2* and *Myog* detected by real-time qPCR were decreased significantly in C2C12 co-cultured on MT1-MMP-deficient BMSCs, compared with that on wild-type BMSCs (Figure 6B). *Mlc2* and *Myog* were expressed in differentiated C2C12 cells but not in BMSCs (Figure 6C). When Notch signalling was inhibited by DAPT, defective myotube formation in C2C12 cells co-cultured on MT1-MMP-deficient BMSCs was rescued (Figure 6A). The decreased mRNA levels of *Mlc2* and *Myog* in C2C12 cells co-cultured on MT1-MMP-deficient BMSCs were partially rescued by low concentration of DAPT (1  $\mu$ M), and completely rescued by high concentration of DAPT (10  $\mu$ M) (Figure 6B), suggesting that the inhibitory effect of MT1-MMP-deficient BMSCs on myotube formation is indeed through Notch signalling. These data further confirm that loss of MT1-MMP activity significantly enhances Notch signalling in neighbouring cells.

### Rescuing impaired bone marrow B-cell development in MT1-MMP conditional knockout mice by Notch signalling inhibitor

To investigate if defective B-cell development in MT1-MMP-deficient mice could be rescued by Notch signalling inhibition, the conditional MT1-MMP-deficient mice were established. (The establishment of conditional MT1-MMP-deficient mice will be reported separately elsewhere.) Prx1Cre transgenic mice were used to delete MT1-MMP in BMSCs (Logan *et al*, 2002; Hilton *et al*, 2008) (Figure 7A). The MT1<sup>flox/flox</sup> Prx1Cre<sup>+</sup> mutant mice survived at least one and a half year. The MT1-MMP protein is nearly completely depleted in the MT1<sup>flox/flox</sup> Prx1Cre<sup>+</sup> BMSCs compared with that in the control MT1<sup>flox/flox</sup> BMSCs (Figure 7B). The B-cell populations in adult mice were then analysed. Consistent with that in MT1-MMP total deficient bone marrow, both early stage (B220<sup>low</sup>CD43<sup>+</sup> CD24<sup>low/-</sup>, B220<sup>low</sup>CD43<sup>+</sup> CD24<sup>high</sup>) and late stage (B220<sup>low</sup>CD43<sup>-</sup>) B cells were reduced in MT1<sup>flox/flox</sup> Prx1Cre<sup>+</sup> bone marrow (Figure 7D–F). MT1-MMP conditional deficient mice (MT1<sup>flox/flox</sup> Prx1Cre<sup>+</sup>) were then received DAPT treatment subcutaneously for 4 weeks. The mRNA level of *Hes5* in HPCs from mock-treated MT1<sup>flox/flox</sup> Prx1Cre<sup>+</sup> bone marrow was much higher than that from control (MT1<sup>flox/flox</sup>) bone marrow, DAPT treatment at concentration of 100 mg/kg body weight significantly reduced *Hes5* transcription, though still higher than that in control (Figure 7C). The reduction in B-cell populations in the conditional deficient bone marrow was largely rescued by Notch inhibitor DAPT, though not to the level similar to that in controls (Figure 7D–F). This is likely due to the incomplete inhibition of Notch reflected by the *Hes5* transcription in HPCs (Figure 7C). These *in vivo* data further support that defective B-cell development in MT1-MMP-deficient bone marrow is attributable, largely if not all, to abnormally enhanced Notch signalling.

### MT1-MMP cleaves Dll1 to inhibit Notch1 signalling

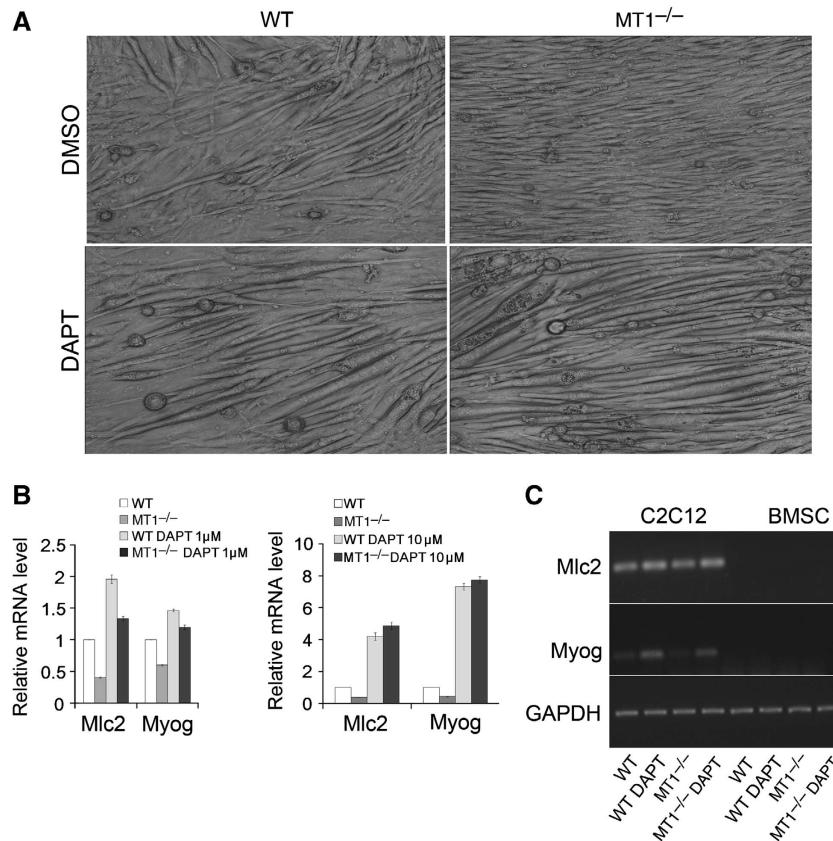
Notch signalling has the unique property that it can be transactivated by the ligands expressed on the surface of neighbouring cells. The enhancement of Notch signalling in HPCs mediated by MT1-MMP-deficient BMSCs might be explained by the increased Notch ligands on the stromal cell surface. Among the five mammalian Notch ligands, Dll1 has been previously shown to induce Notch signalling, leading to blockage of B-cell development (Schmitt and Zuniga-Pflucker, 2002). The cleavage of Dll1 inhibits Notch signalling (Muraguchi *et al*, 2007). Similar to Dll1, the metalloproteinase MT1-MMP is also expressed on membrane,



**Figure 5** MT1-MMP-deficient BMSCs partially block B-cell development *in vitro* in a Notch signalling-dependent manner. (A) Immunoblot analyses of aNotch1 in lineage-negative Sca1<sup>+</sup> cKit<sup>+</sup> HPCs co-cultured on MT1-MMP<sup>-/-</sup> BMSCs compared with those co-cultured on wild-type BMSCs. aNotch1 in the wild-type HPCs without *in vitro* culture is used as a control. β-Actin serves as an internal loading control. (B) Real-time PCR analyses of mRNA levels of *Hes1* and *Hes5* in HPCs co-cultured on MT1-MMP<sup>-/-</sup> BMSCs compared with those co-cultured on wild-type BMSCs;  $P = 0.0091$  (*Hes5*), Student's *t*-test. Data are representative of three independent experiments (mean ± s.d.). (C) Flow cytometry analyses of lymphocyte commitment from lineage-negative Sca1<sup>+</sup> cKit<sup>+</sup> HPCs co-cultured on either wild-type BMSCs or MT1<sup>-/-</sup> BMSCs. Both are treated with either DMSO or Notch inhibitor DAPT. (D) Statistical analyses of B-cell commitment from co-cultured HPCs in (C);  $P = 0.00756$  (WT DMSO vs MT1-MMP<sup>-/-</sup> DMSO),  $P = 0.0278$  (MT1-MMP<sup>-/-</sup> DMSO vs MT1-MMP<sup>-/-</sup> DAPT), Student's *t*-test. Data are representative of two independent experiments (mean ± s.d.). (E, F) Flow cytometry analyses of T-cell commitment from lineage-negative Sca1<sup>+</sup> cKit<sup>+</sup> HPCs co-cultured on either wild-type or MT1-MMP-deficient BMSCs. Both are treated with either DMSO or Notch inhibitor DAPT. Numbers in the plots indicate CD4 SP (bottom-right), CD8 SP (up-left), CD4CD8 DP (up-right) and DN (bottom-left) cells in (E) or CD25<sup>+</sup>CD44<sup>+</sup> DN T-cell progenitors (up-right) in (F).

raising the possibility that MT1-MMP might promote the cleavage of Dll1 in BMSCs to inhibit Notch signalling in neighbouring HPCs. To test this hypothesis, we first examined the interaction between MT1-MMP and Dll1 in BMSCs and found that these two proteins were co-immunoprecipitated each other (Figure 8A). In addition, the full-length Dll1 (Dll1-FL) was significantly less in the wild-type BMSCs cells than that in the MT1-MMP-deficient BMSCs, while there was an increased accumulation of an ~30-kDa C-terminal fragment

of Dll1 (Dll1-CTF) in the wild-type BMSCs (Figure 8B; Supplementary Figure S5A), suggesting that MT1-MMP promotes the cleavage of Dll1 in BMSCs. Knocking-down of MT1-MMP in wild-type BMSCs again resulted in significantly reduced Dll1-CTF and increased full-length Dll1 (Figure 8B; Supplementary Figure S5A). Jagged1 (*Jag1*), another Notch ligand reported to be expressed in BMSCs, was examined and no difference were found between wild-type and MT1-MMP-deficient BMSCs (Figure 8C). Dll4 is highly expressed in



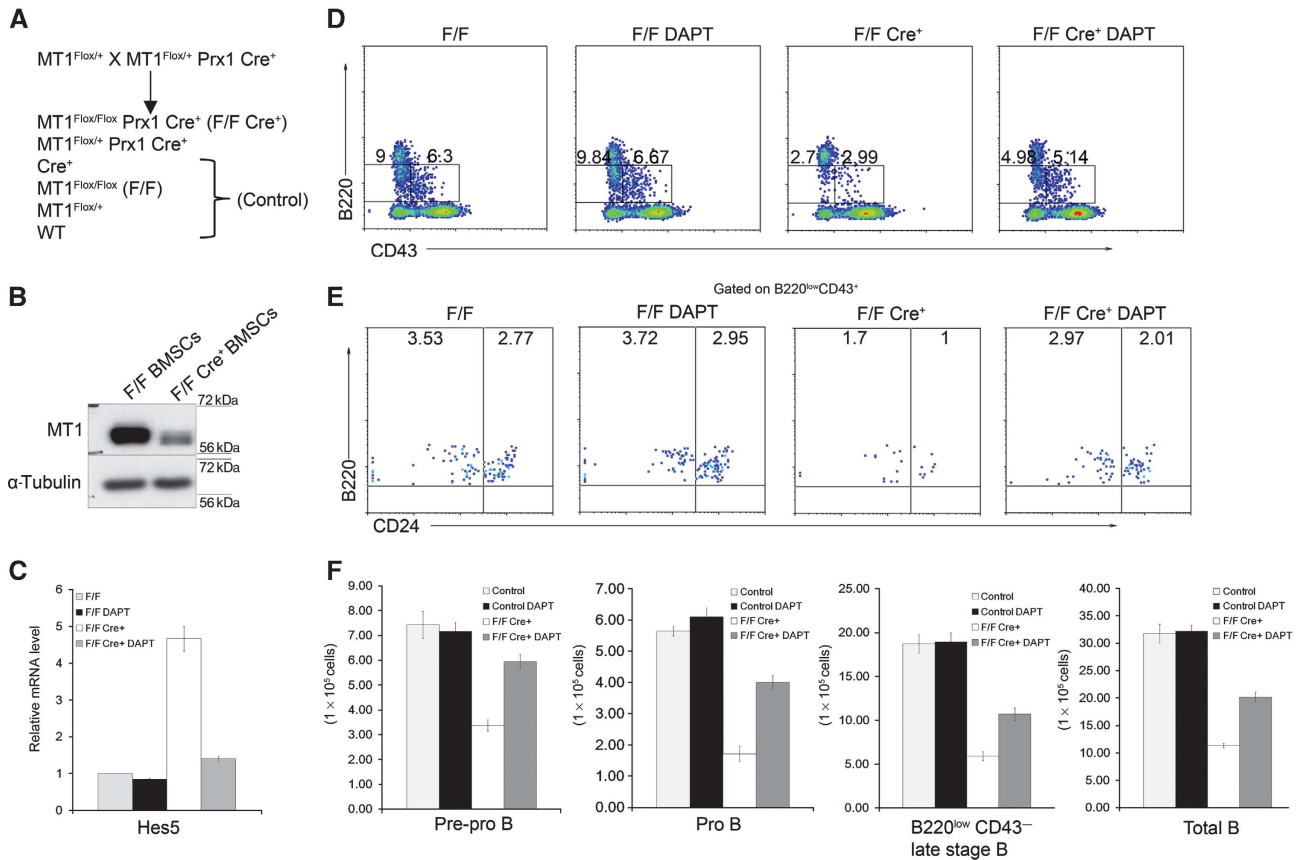
**Figure 6** MT1-MMP-deficient BMSCs inhibit myotube formation of C2C12 cells in a Notch signalling-dependent manner. (A) Myotube formation analyses of C2C12 myoblasts co-cultured on either wild-type or MT1-MMP<sup>-/-</sup> BMSCs are shown. Both are treated with either DMSO or Notch inhibitor DAPT. Pictures are taken after 2 weeks of differentiation induction with a lower concentration of horse serum. Original magnification,  $\times 200$ . (B) Real-time PCR analyses of *Mlc2* and *Myog* mRNA levels in the C2C12 cells co-cultured on either wild-type or MT1-MMP<sup>-/-</sup> BMSCs, treated with either DMSO or different dose of DAPT. Data are representative of two independent experiments (mean  $\pm$  s.d.). (C) Semi-quantitative RT-PCR analyses of *Mlc2* and *Myog* expression in C2C12 cells prepared in (A) and BMSCs with the same culture condition.

thymus epithelial cells and affects the T-cell development in the thymus (Hozumi *et al*, 2008; Koch *et al*, 2008). However, its expression was not detectable in BMSCs. Furthermore, Dll4 was not cleaved by MT1-MMP when they were co-expressed (Figure 8F).

We then ectopically expressed wild-type human MT1-MMP (MT1FL) and active-site mutant MT1-MMP (MT1EA), respectively, in HEK293 cells together with mouse Dll1. As shown in Figure 8D, expression of wild-type MT1-MMP resulted in reduced full-length Dll1 and increased Dll1-CTF fragment, compared with that in cells expressing mutant MT1-MMP (Figure 8D). To further demonstrate that the increased cleavage of Dll1 mediated by MT1-MMP would lead to inactivation of Notch signalling in neighbouring cells, we established a 293 cell line stably expressing human Notch1 (293hN1) and HeLa cell lines that ectopically express either wild-type or activity-mutated MT1-MMP, together with Dll1. Similar to our observations in HEK293 cells, an increased cleavage of Dll1 was observed in HeLa cells expressing wild-type MT1-MMP, compared with that in HeLa cells expressing mutant MT1-MMP. When 293hN1 cells were seeded on top of HeLa cells expressing MT1-MMP and Dll1, significantly reduced Notch1 signalling was observed in 293hN1 cells co-cultured with HeLa cells expressing wild-type MT1-MMP, compared with that in 293hN1 cells co-cultured with HeLa cells expressing mutant

MT1-MMP (Figure 8E). These results further demonstrate that MT1-MMP can promote the cleavage of the Notch ligand Dll1 and the enhanced cleavage of Dll1 mediated by MT1-MMP inhibits Notch signalling in neighbouring cells. Thus, lack of MT1-MMP diminishes the lateral inhibitory effect and leads to enhanced Notch signalling in neighbouring cells. This explains the abnormal B-cell development in bone marrow of MT1-MMP-deficient mice, even though neither HPCs nor B cells expresses MT1-MMP.

The cleavage of Dll1 produces an  $\sim 30$ -kDa C-terminal fragment, similar to that cleaved by ADAMs (Six *et al*, 2003). It has been suggested that the cleavage site of Dll1 by ADAM10 is at H<sup>-536</sup>M. To determine if MT1-MMP cleaves Dll1 directly, we synthesized a Dll1 polypeptide (<sup>516</sup>QFLLPEP PPGPMVVDLSERHMESQGGPFP) containing ADAM10 cleavage site H<sup>-536</sup>M and incubated it with the recombinant catalytic form of MT1-MMP. Mass spectrometry analyses of the reaction products showed a fragment at 2015.9 Da (Supplementary Figure S6A). Its amino-acid sequence was further identified as <sup>527</sup>MVVDLSERHMESQGGPFP by tandem MS/MS (Supplementary Figure S6B), suggesting that MT1-MMP can cleave Dll1 directly at P<sup>-527</sup>M. This cleavage site is different from that by ADAM10. We then purified the  $\sim 30$ -kDa C-terminal fragment from HEK293 cells expressing both wild type MT1-MMP and Dll1 for N-terminal sequencing by Edman degradation. Again, a Dll1 fragment started



**Figure 7** Rescuing impaired bone marrow B-cell development in MT1-MMP conditional deficient mice by Notch signalling inhibitor. (A) The strategy to breed MT1-MMP conditional deficient MT1<sup>Flox/Flox</sup> Prx1Cre<sup>+</sup> mice. (B) Immunoblot analyses of MT1-MMP in BMSCs purified from MT1-MMP conditional deficient mice and littermate controls. (C) Real-time PCR analyses of *Hes5* mRNA level in isolated HPCs from MT1-MMP conditional deficient mice and littermate controls, treated with either Notch inhibitor DAPT or solvent only. (D, E) Flow cytometry analyses of bone marrow B-cell populations in MT1-MMP conditional deficient and littermate control mice treated with either DAPT or solvent only. Numbers adjacent to the outlined areas in (D) indicate the percentage of early stage B220<sup>+</sup> CD43<sup>+</sup> cells (right) or late stage B220<sup>+</sup> CD43<sup>-</sup> cells (left). Numbers in the plots in (E) indicate the percentage of B220<sup>+</sup> CD43<sup>+</sup> CD24<sup>low/-</sup> cells (left) or B220<sup>+</sup> CD43<sup>+</sup> CD24<sup>high</sup> cells (right). (F) Statistical analyses of B-cell populations in MT1-MMP conditional deficient mice and littermate controls, treated with DAPT or solvent only. Data are representative of two independent experiments (mean ± s.d.). Five to six mice are analysed for each group.

from <sup>527</sup>M was identified (Supplementary Figure S7A and B), confirming the existence of a novel Dll1 cleavage site at P-<sup>527</sup>M by MT1-MMP. We also identified the Dll1 fragment started from <sup>536</sup>M, which is likely produced by endogenous ADAMs.

## Discussion

MT1-MMP is a membrane-bound matrix metalloproteinase first identified on the surface of invasive tumour cells as an activator of proMMP2 (Sato *et al*, 1994). MT1-MMP contributes to the degradation of various extracellular matrix (ECM) components and thus promotes invasion and migration (Seiki and Yana, 2003). However, the role of MT1-MMP is broader than simply degradation of ECM components. It has also been shown to cleave membrane receptors and ligands and consequently regulate intracellular signal transduction (Lehti *et al*, 2005, 2009). Here, we show that MT1-MMP cleaves Dll1 in BMSCs to inhibit Dll1-induced Notch signalling in neighbouring HPCs, and thus maintains normal B-cell development in bone marrow (Figure 9).

Notch signalling provides direct cell-cell communication through ligand-receptor interaction-mediated signalling

transduction. The wide expression of Notch ligands on stromal cells and receptors on haematopoietic cells indicates an important function of Notch signalling in haematopoiesis (Radtke *et al*, 2004). Indeed, Notch signalling has been shown to promote T-cell development but block B-cell development through gain of function studies *in vivo* and *in vitro* (Pui *et al*, 1999; Schmitt and Zuniga-Pflucker, 2002). However, in bone marrow, only B lymphocytes are generated, indicating that bone marrow Notch signalling is not sufficient to initiate T-cell commitment. In addition, loss of function in Notch signalling does not disturb bone marrow lineage development (Radtke *et al*, 1999), suggesting that proper level of Notch signalling in bone marrow is critical in maintaining B-cell development. Notch signalling is also essential for the formation of embryonic haematopoietic stem cells (HSCs; Kumano *et al*, 2003; Robert-Moreno *et al*, 2008). Gain of function analyses, including activating Notch signalling *in vivo* or treating HSCs with ligands *in vitro*, support the idea that Notch signalling promotes survival and self-renewal of HSCs (Varnum-Finney *et al*, 1998; Karanu *et al*, 2000, 2001; Stier *et al*, 2002; Burns *et al*, 2005; Suzuki *et al*, 2006). However, inactivation of Notch signalling, by either over-expressing dominant-negative Mastermind-like 1 or deletion





specific gene expressions of *Myog* and *Mlc2* in C2C12 on MT1-MMP-deficient BMSCs are completely rescued by DAPT. The enhanced Notch signalling resulted from MT1-MMP deficiency is due to the impaired processing of Dll1 on the cell surface and MT1-MMP activity is required for the efficient processing of Dll1. Importantly, recombinant MT1-MMP cleaves Dll1 peptide at the same site where MT1-MMP cleaves Dll1 on cell surface identified by N-terminal Edman sequencing. Thus, MT1-MMP-mediated Dll1 cleavage on stromal cells has an important role in the negative regulation of Notch signalling in bone marrow.

The T-cell development in MT1-MMP-deficient bone marrow is not significantly affected. Previous studies have shown that the threshold of Notch signalling to promote T-cell development is higher than that to block B-cell development (Schmitt *et al*, 2004). Therefore, it is likely that the increased Notch signalling resulted from the MT1-MMP deficiency is not sufficient to initiate T-cell development, though sufficient to affect B-cell differentiation. It is unclear why the enhancement of Notch signalling in MT1-MMP-deficient mice is mild. One possibility is due to the moderate level of Dll1 expression in BMSCs. In addition, Dll1 is cleaved by some membrane metalloproteinases other than MT1-MMP, such as ADAM9 and ADAM10 (Six *et al*, 2003; Dyczynska *et al*, 2007). Both of them are expressed in BMSCs (Supplementary Figure S8). This is consistent with the existence of Dll1-CTF in MT1-MMP-deficient BMSCs (Figure 8B). The cleavage by ADAMs may partially compensate for the loss of MT1-MMP and minimize the activation of Notch signalling in MT1-MMP-deficient mice. Furthermore, the negative regulation of LRF on Notch signalling may also reduce the enhancement of Notch signalling in MT1-MMP-deficient mice. However, LRF is not able to completely suppress the enhanced Notch signalling in MT1-MMP-deficient mice. It is worth of further investigation whether the function of LRF is disturbed in MT1-MMP-deficient mice.

The T-cell development in thymus is normal and the protein level of aNotch1 in thymus is comparable between wild-type and MT1-MMP-deficient mice (Supplementary Figure S9A), indicating that Notch signalling is not affected by MT1-MMP in the thymus. This is in line with the fact that expression of MT1-MMP is minimal in thymus. Similarly, it may also explain the normal B-cell populations and unaltered aNotch1 level in MT1-MMP mutant fetal liver (Supplementary Figure S9B). These data indicate that MT1-MMP may affect B-cell development specifically through bone marrow niches.

B cells are derived from bone marrow HSCs through a serial stage of intermediate progenitor cells. The primitive HSCs have been reported to locate in different bone marrow microenvironment niches, composed of blood vessels, osteoblasts or stromal cells (Kiel *et al*, 2005; Sugiyama *et al*, 2006). To differentiate into B cells, the intermediate HPCs are recruited to BMSCs (Tokoyoda *et al*, 2004) where MT1-MMP is highly expressed. MT1-MMP is also expressed in osteoblasts, endothelial cells and vascular smooth muscle cells (Kinoh *et al*, 1996; Lehti *et al*, 2005; Yana *et al*, 2007), indicating that MT1-MMP may also affects the osteoblastic niches and vascular niches of HSCs. However, the HSCs population in MT1-MMP-deficient bone marrow is normal (data not shown), suggesting that the maintenance of HSCs population might not be affected by MT1-MMP deficiency-

triggered Notch signalling enhancement. Similarly, the deficiency of LRF in bone marrow also results in enhanced Notch signalling but normal population of HSCs (Maeda *et al*, 2007). It is not clear whether much higher Notch signalling is required to disturb the maintenance of HSCs, though it seems to be the case for T-lymphocyte commitment in bone marrow.

The increased Notch signalling in MT1-MMP-deficient mice does not result in overall phenotypes similar to that observed in transgenic mice overexpressing aNotch1. This could be due to the restrict expression of MT1-MMP. Similar phenotypes may only be observed in tissues where both MT1-MMP and Notch signalling are prominently expressed. In addition, the level of Notch signalling may also be responsible for the difference in the phenotypes between MT1-MMP-deficient mice and aNotch1 transgenic mice.

Our data suggest that MT1-MMP directly cleaves Notch ligand Dll1 in BMSCs. The cleavage produces an ~30-kDa C-terminal fragment of Dll1, a size similar to the Dll1 cleaved by ADAMs (Six *et al*, 2003; Dyczynska *et al*, 2007). Recombinant MT1-MMP directly cleaves Dll1 peptide at the site close to but different from that cleaved by ADAMs. Interestingly, Dll1-CTF can be found in MT1-MMP-deficient BMSCs though much less compared with that in wild-type BMSCs (Figure 8B), suggesting that at least in BMSCs, MT1-MMP is the major negative regulator of Dll1-mediated Notch signalling. As Jagged1 is not affected by MT1-MMP and Dll4 is undetectable by RT-PCR in MT1-MMP-deficient BMSCs, the enhanced Notch signalling in MT1-MMP-deficient HPCs is mostly attributable to the defective cleavage of Dll1 in BMSCs. In conclusion, MT1-MMP is required specifically for B-lymphocyte development by fine-tuning Notch signalling in HPCs through regulating Notch ligand Dll1 on BMSCs. The regulation of Notch signalling by MT1-MMP is tissue dependent. Our data not only demonstrate for the first time that MT1-MMP negatively modulates Notch signalling to maintain normal B-lymphocyte development but also provide new insights into various developmental processes regulated by the interplay between MT1-MMP and Notch signalling.

## Materials and methods

### Mice and cell culture

MT1-MMP-deficient mice were established previously (Zhou *et al*, 2000). Sex matched littermates sacrificed at 10–14 days old were used for all experiments. To generate mouse BMSCs, total bone marrow cells were isolated from long bones (both femur and tibia) and cultured in  $\alpha$ MEM medium supplemented with 9% fetal bovine serum (FBS; Gibco) and 9% horse serum (HS; Hyclone) (Peister *et al*, 2004). Medium was replaced every 3–4 days to remove non-adherent haematopoietic cells. After 2 weeks, the adherent cells were collected and sorted with a mouse lineage kit (BD Bioscience) and anti-biotin magnetic beads (Miltenyi Biotech). The sorted BMSCs were cultured in fresh medium and further subcultured. The first passage of sorted BMSCs was termed passage 1. BMSCs between passages 7 and 12 were used for experiments unless otherwise stated.

### DNA constructs

The original human MT1-MMP cDNA was kindly given by Dr Pei (University of Minnesota). The MT1-MMP cDNA was subcloned into pTracer-CMV vector (Invitrogen). The construct expressing inactive MT1-MMP was made through mutating E240 to A. The full-length mouse Dll1 cDNA was obtained by PCR from a mouse BMSC cDNA library and cloned into pcDNA3.1 vector tagged with Flag at the C-terminus. The human Notch1 cDNA was obtained from Artavanis-Tsakonas Laboratory (Harvard Medical School).

### Flow cytometry analysis

Total bone marrow cells were isolated from long bones and stained with fluorescence-conjugated antibodies in HBSS supplemented with 2% FBS. The cells were fixed and the red blood cells were lysed using BD FACS lysing solution (BD Bioscience) after staining and then processed for flow cytometry analyses. *In vitro* cultured cells were collected and stained with fluorescence-conjugated antibodies in HBSS supplemented with 2% FBS. The cells were either fixed with 4% (wt/vol) paraformaldehyde or directly processed for flow cytometry after staining. FITC anti-CD45.2 (109805), PE/Cy5 anti-B220 (103210), PE anti-CD3 (100205), FITC anti-CD4 (100406), PE/Cy5 anti-CD8 (100710) and PE anti-CD44 (103023) were purchased from BioLegend. PE anti-CD43 (553271), FITC anti-CD24 (553261) and FITC anti-CD25 (558689) were purchased from BD Pharmingen.

### Bone marrow reconstitution

Total bone marrow cells were isolated from the MT1-MMP-deficient mice and comparable wild-type mice with CD45.2 background. The cells were transplanted into lethally irradiated adult mice with CD45.1 background. After 2 months, the transplantation efficiency was examined by flow cytometry analysis of donor CD45.2 cells and recipient CD45.1 cells. Six months later, lymphocyte development was examined by flow cytometry analysis after the bone marrow cells were stained with FITC anti-CD45.2, PE/Cy5 anti-B220, and PE anti-CD3. FITC anti-CD45.1 (110705) was purchased from BioLegend.

### Immunoblot analysis

Cells were lysed in a lysis buffer composed of 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate and protease inhibitor cocktail (Roche). Protein lysate was mixed with loading buffer, separated by SDS-PAGE and then analysed by immunoblot analysis. Monoclonal anti-MT1-MMP (ab51074) was purchased from Abcam. Polyclonal anti-cleaved Notch1 (2421) was purchased from Cell Signaling. Polyclonal anti-Dll1 (sc-8155 and sc-9102) was purchased from Santa Cruz. Monoclonal anti-Flag (F1804), monoclonal anti- $\beta$ -actin (A5316) and monoclonal anti- $\alpha$ -tubulin (T5168) were purchased from Sigma.

### Semi-quantitative RT-PCR and real-time PCR analysis

Total RNA was extracted from cells, and single complementary DNA (cDNA) was synthesized using Promega M-MLV reverse transcriptase (M170A). After mixing with specific primers, dNTP and Finnzymes DNA polymerase (F501L), semi-quantitative PCR was performed with ABI 9700 PCR system. To perform Real-time PCR, cDNA was mixed with specific primers and ABI Power SYBR Green PCR Master Mix (4367659). The reaction was performed with ABI StepOnePlus Real-time PCR system.

### Co-immunoprecipitation

Cell lysate was pre-cleared with protein A or G agarose (Roche) and then incubated with rabbit anti-MT1-MMP antibody (ab51074, Abcam) or goat anti-Dll1 antibody (sc-8155, Santa Cruz) for 2 h at 4°C with agitation. The mixture was then incubated with protein A or G agarose overnight at 4°C with agitation. The agarose beads were washed three times with lysis buffer and boiled in loading buffer. The precipitated protein was analysed by immunoblot analysis.

### Immunohistochemistry

The long bone was fixed in 4% (wt/vol) paraformaldehyde, dehydrated through serial ethanol and embedded in paraffin. Paraffin sections were cut at a thickness of 6  $\mu$ m and then processed for MT1-MMP staining.

### C2C12 myotube formation assay

Either wild-type or MT1-MMP-deficient BMSCs were seeded in 24-well culture plates. When the cells became confluent, C2C12 myoblasts were plated on the BMSCs at a density of  $5 \times 10^3$ /well and maintained in DMEM supplemented with 10% FBS. The

medium was replaced with DMEM supplemented with 2% HS after 24 h to induce myotube formation.  $\gamma$ -Secretase inhibitor, DAPT (Sigma), and comparable DMSO were added to selected cultures. The medium was replaced every 3–4 days. Myotube formation was monitored after 2 weeks.

### In vitro lymphocyte development assay

Either wild-type or MT1-MMP-deficient BMSCs were seeded in 24-well culture plates. When the cells became confluent, sorted lineage-negative Scd1<sup>+</sup>cKit<sup>+</sup> HPCs from adult mice total bone marrow were plated on top of the BMSCs at a density of  $4 \times 10^3$  and maintained in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate,  $2 \times 10^{-5}$  M 2-ME and 5 ng/ml Flt-3L and IL-7 (R&D).  $\gamma$ -Secretase inhibitor, DAPT, and comparable DMSO were added to selected cultures. Lymphocyte commitment from HPCs was analysed by flow cytometry analysis at selected time points.

### In vivo DAPT treatment

DAPT was dissolved in 90% corn oil in ethanol and administered subcutaneously at a dose of 100 mg/kg body weight, daily for 3 days in a week (Hellstrom *et al*, 2007; Maeda *et al*, 2007). The mice were treated for 4 weeks before they were sacrificed for analysis.

### Mass spectrometry and Edman degradation

The polypeptide of mouse Dll1 (<sup>516</sup>QFLLEPPPGPMVVDLSERH-MESQGGPPF) was synthesized (GL Biochem) and incubated with recombinant catalytic domain of MT1-MMP (BML-SE259) in the digestion buffer composed of 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 10 mM CaCl<sub>2</sub>. After incubation for 16 h at 37°C, the reaction products were analysed by mass spectrometry using an ABI4800 MALDI TOF/TOF™ Analyzer. To determine the N-terminal sequence of Dll1-CTE, the Flag-tagged fragment was purified from 293 cells overexpressing MT1-MMP and Dll1 using flag purification kit (CELLMM2, Sigma), blotted onto the PVDF membrane and then processed to Edman degradation (Shanghai GeneCore BioTechnologies Co., Ltd.).

### Statistical analysis

Statistical significance was identified with Student's *t*-test or one-way ANOVA. *P*-values of <0.05 were considered statistically significant.

### Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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*Author contributions:* GJ performed most of the experiments. GJ, KMC and KSEC made the conditional MT1-MMP-deficient mice. FZ, HLW, BL, XL and DL helped with some experiments. GJ, KC, CM and ZZ analysed the data. GJ and ZZ prepared the manuscript.

## Conflict of interest

The authors declare that they have no conflict of interest.

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