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MECHANISMS OF DEVELOPMENT

Three matrix metalloproteinases are required *in vivo* for macrophage migration during embryonic development

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ABSTRACT

Macrophages are essential in development, repair and pathology of a variety of tissues via their roles in tissue remodelling, wound healing and inflammation. These biological functions are also associated with a number of human diseases, for example tumour associated macrophages have well defined functions in cancer progression. *Xenopus* embryonic macrophages arise from a haematopoietic stem cell population by direct differentiation and act as the main mechanism of host defence, before lymphoid cells and a circulatory system have developed. This function is conserved in mouse and human development. Macrophages express a number of matrix metalloproteinases (MMPs), which are central to their function. MMPs are a large family of zinc-dependent endoproteases with multiple roles in extracellular matrix remodelling and the modulation of signalling pathways. We have previously shown MMP-7 to be expressed by *Xenopus* embryonic macrophages. Here we investigate the role of MMP-7 and two other MMPs (MMP-18 and MMP-9) that are also expressed in the migrating macrophages. Using morpholino (MO) mediated knockdown of each of the MMPs we demonstrate that they are necessary for normal macrophage migration *in vivo*. The loss-of-function effect can be rescued using the specific MMPs, altered to be resistant to morpholinos but not by overexpression of the other MMPs. Double and triple morpholino knockdowns further suggest that these MMPs act combinatorily to promote embryonic macrophage migration. Thus, our results imply that these three MMPs have distinct functions, which together are crucial to mediate macrophage migration in the developing embryo. This demonstrates conclusively that MMPs are required for normal macrophage cell migration in the whole organism.

1. Introduction

The MMPs are a large multigene family of 24 secreted and cell surface enzymes in mammalian genomes that share common functional domains and activation mechanisms. They are important for cell migration, invasion, proliferation and apoptosis and regulate many developmental processes, including branching morphogenesis, angiogenesis, extracellular matrix degradation and wound healing (Page-McCaw et al., 2007; Vu and Werb, 2000).

MMP substrates include many structural extracellular matrix (ECM) proteins, other proteinases, proteinase inhibitors, clotting factors, growth factors, cell surface receptors and cell–cell adhesion molecules (Somerville et al., 2003; Sternlicht and Werb, 2001). Recent evidence has implicated MMPs in the regulation of cell survival, angiogenesis, inflam-

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mation, signalling and wound healing and often correlates with remodelling of the ECM. As a result of their potent proteolytic activity, abnormal MMP function can also contribute to pathological conditions such as atherosclerosis, tumour metastasis and diseases of the nervous system such as multiple sclerosis, malignant gliomas and Alzheimer's disease (Galis and Khatri, 2002; Page-McCaw et al., 2007; Platt et al., 2003; Yong et al., 2001).

Many cell based studies suggest roles for MMPs in cell migration (Hotary et al., 2006; Stringa et al., 2000) but a major challenge in understanding the biology of MMPs is the characterisation of their function(s) in the whole organism. To date little is known concerning the distribution and/or role of MMPs in developing embryos. Mouse knockouts of MMPs and their natural inhibitors, the TIMPs, have suggested that certain MMPs are involved in development through degradation of ECM as well as liberation of growth factors associated with ECM components (Holmbeck et al., 1999; Sternlicht and Werb, 2001). However, often MMP null mice show minor or subtle phenotypes possibly due to redundancy between family members (Milner and Cawston, 2005). In some cases a phenotype only becomes apparent when the mice are challenged in various ways. To date many of these challenges have been in disease related models (Milner and Cawston, 2005). For instance, MMP-7 (matrilysin) has been shown to be necessary for neutrophil influx into sites of inflammation in mice using a well characterised model for lung fibrosis (Li et al., 2002). Thus far the mouse has been the organism of choice to study the role of MMPs in whole animals in particular in disease models. Studies in other organisms such as chick, zebrafish, *Xenopus* and *Drosophila* have however also suggested that MMPs play an important role in tissue morphogenesis during normal development (Cai and Brauer, 2002; Jung et al., 2002; Page-McCaw et al., 2003; Zhang et al., 2003).

A number of *Xenopus* MMPs have been described by ourselves and others including MMP-2, -7, -9, -11, -13, -14, -15, -16, -18 and -24 (Carinato et al., 2000; Damjanovski et al., 2001; Harrison et al., 2004; Jung et al., 2002; Stolow et al., 1996; Yang and Kurkinen, 1998). Many of these are expressed in the developing embryo. However, very little is known about their function. Overexpression experiments in which MMP-11 (ST3), MMP-18 and mouse MT-MMP5 (MMP-24) were ubiquitously expressed during *Xenopus* development led to lethality during late embryonic development (Damjanovski et al., 2001). Further overexpression experiments have shown that MMP-2 and MMP-14 act co-operatively during *Xenopus* development (Hasebe et al., 2007). To date no experiments have been done looking at a loss-of-function of MMPs during *Xenopus* development.

As part of our initial experiments identifying MMPs in early *Xenopus* embryos we found some of them to be expressed in embryonic macrophages (Harrison et al., 2004). Embryonic macrophages (phagocytic myeloid cells) provide innate immunity in early developing embryos until the adaptive immune system appears which in *Xenopus* tadpoles is around day 12 (Turpen, 1998). The innate immune system is of fundamental importance to survival in hostile aquatic environments. The amphibian embryonic macrophage cells bear a remarkable similarity to embryonic and fetal macrophages found in higher vertebrates including man (Shepard and Zon, 2000). In addition to a role in immunity these cells also have an important role to play in embryogenesis by clearing apoptotic cells, for instance in the region between the developing digits (Hopkinson-Woolley et al., 1994). In mice and birds fetal macrophages appear in the yolk sac well before any monocytes or granulocytes, suggesting that they arise from a haematopoietic stem cell through a pathway that by passes the monocytic series. So called 'primitive macrophages' mature quickly into 'fetal macrophages' (Takahashi et al., 1996). From the yolk sac they quickly invade the mesenchyme of the head and other forming organs (Cuadros et al., 1993; Sorokin et al., 1992). In zebrafish early macrophages with an ability to divide have been described arising from the ventro-lateral mesoderm (Herbomel et al., 1999, 2001). In *Xenopus*, embryonic macrophages arise in the anterior ventral blood island (VBI) where the embryonic haematopoietic cell lineage arises (Harrison et al., 2004; Smith et al., 2002; Tashiro et al., 2006).

Xenopus is very well suited for *in vivo* analysis of cell migration (DeSimone et al., 2005) and has been a classic model for studies on cell movements and migration during gastrulation (Keller et al., 2003). In this paper we show that *Xenopus* is also a highly amenable model system to study individual cell migration. We focus on functional analysis of the MMPs using a morpholino knockdown approach (Heasman, 2002). We show that MMPs-7, -9 and -18 are critical for embryonic macrophage migration and that they work together in a non-redundant fashion to do this. This significantly contributes to our understanding of the specific function of these proteins in the whole organism.

2. Results

2.1. *Expression of three MMPs is restricted to the migrating embryonic macrophages*

Xenopus POX2, a member of the peroxidase family, is a specific marker for myeloid cells (Smith et al., 2002), that are functional macrophages in the embryo (Lichanska and Hume, 2000; Shepard and Zon, 2000). These POX2 positive cells co-localise with the leukocyte specific proteins L-Plastin (Smith et al., 2002) and XL-1 (Ohinata et al., 1989) and exhibit the macrophage typical behaviour of phagocytosis (Smith et al., 2002). Embryonic macrophages are first observed in the VBI at stage 17–18 and at stage 20 begin to migrate over the whole embryo between the epidermis and the mesoderm (Harrison et al., 2004; Smith et al., 2002), completing their migration by stage 25. This process can easily be detected and scored visually across the whole embryo. We have previously shown that MMP-7 is expressed in macrophages where it co-localises with POX2 (Harrison et al., 2004). Further analysis of *Xenopus* MMPs identified MMP-9 (Carinato et al., 2000) and MMP-18 (Dr. J. Henry personal communication, see http://www.life.uiuc.edu/henry/lab_db.php?cid=L080) to be co-expressed with POX2 as well. Expression patterns of these three MMPs are shown at the early phase of macrophage migration (Fig. 1A–C). The initial radial dispersion pattern away from the VBI and co-localisation of MMP expression with POX2 in single macrophages is shown by double *in situ* hybridisation.