FEBS Letters 581 (2007) 4960–4964

FGF mediated *Sulf1* regulation

Wanfeng Zhao, Steve Allen, Gurtej K. Dhoot*

Department of Basic Sciences, The Royal Veterinary College, University of London, Royal College Street, London NW1 OTU, UK

Received 15 August 2007; revised 6 September 2007; accepted 11 September 2007

Available online 24 September 2007

Edited by Lukas Huber

Abstract FGF signalling is critical for normal embryonic development. *Sulf1* has been shown to inhibit FGF activity. The role of FGF4 in *Sulf1* regulation was investigated during digital development of the quail autopod. Implantation of FGF4 beads in both the interdigit and at the tip of digit III differentially up-regulated *Sulf1* as also confirmed in micromass cultures. FGF4 inhibited interdigital mesodermal apoptosis in a concentration dependent manner. The FGF inhibitor, SU5402, inhibited *Sulf1* expression when placed in the interdigital mesoderm. However, when placed at the digital tip, SU5402 induced an ectopic domain of *Sulf1* expression and inhibited further phalange formation.

Keywords: *Sulf1*; Digit; Interdigit; FGFs; Apoptosis; Micromass

1. Introduction

The specific activities of growth factors and signalling molecules regulate cell fate determination, proliferation, differentiation and programmed cell death of mesodermal cell populations in the developing limb. Cell signalling can be modulated through the regulation of ligand and receptor expression. Equally important in these processes, however, are the secondary receptors and other factors that modulate the activities of such molecules since it is the balance of their overall activities that determines the final outcomes. One such factor that was discovered in recent years is *Sulf1*, a member of the sulfatase family of enzymes [1–3] that can regulate the activities of many growth factors and signalling molecules [4,5]. Modulation occurs by regulating the sulfation status of specific heparan sulfate proteoglycans (HSPGs) required for growth/signalling factor function. For example, *Sulf1* has been shown to regulate the activities of Wnts [4,6], BMPs (bone morphogenic protein) [7] and fibroblast growth factors (FGF) in vertebrates. FGF activity has been shown to be inhibited by *Sulf1* during angiogenesis in the chick, and also in ovarian cells, by removing 6-*O* sulfates from glucosamine residues in heparin sulfate of HSPGs. These sulfated HSPGs act as secondary receptors required for FGF2 and FGF4 function [5,6,8,9]. Since *Sulf1* has the potential to modulate the activities of FGFs negatively, we investigated the effect of FGF4 on the expression of *Sulf1* in both undifferentiated interdigital mesoderm and condensed (differentiating) digital mesoderm that gives rise to digital rays. These two mesodermal populations have different cell fates. The condensed digital mesoderm is destined to become chondrogenic and form the digital rays while the interdigital mesoderm undergoes apoptosis.

Although FGFs have long been known to promote mesodermal proliferation [10], studies in the developing autopod have also demonstrated a role in the regulation of cell death. The precise role of FGFs in this process, however, is far from clear since both positive and negative FGF regulation has been reported to inhibit BMP induced apoptosis. For example, Montero et al. [11] have shown that FGFs co-operate with BMPs in the control of mesodermal apoptosis while disruption of FGF in a mouse model has been demonstrated to lead to digital syndactyly due to an inhibition of cell death [12]. Some further observations regarding the role of FGF signalling in the development of autopodal mesoderm, however, have been controversial. For example, while interdigital cells in the chick have been shown to respond to FGF4 by inhibiting apoptosis resulting in interdigital webbing, in the mouse only a transient decline in apoptosis is seen having no effect on digit separation [13]. Our present study of the developing autopod showed *Sulf1* to be up-regulated in response to FGF4. The effects of FGF4 on apoptosis, however, were concentration dependent since moderate, but not high or low, levels of FGF4 inhibited apoptosis resulting in syndactly. Implantation of beads containing high levels of FGF4, however, did result in an inhibition of apoptosis in the adjoining interdigital mesoderm suggesting that the FGF signal formed a gradient within the tissues of the autopod.

2. Materials and methods

2.1. Surgical procedures

Fertilized quail eggs incubated at 38 °C were used for all bead implantations at developmental stage 27–28. For bead implantation in the interdigital mesoderm, a fine slit was made between digits III and IV using a sterile tungsten needle before insertion of an FGF4 or FGF inhibitor (SU5402) soaked bead. For bead implantation at the tip of digital ray III, a fine slit was made in the digital tip before insertion of FGF4 or SU5402 soaked beads. Embryos were re-incubated at 38 °C and harvested 24 or 72 h post-surgery. For bead preparation, Heparin acrylic beads (Sigma), size range 100–150 μm in diameter, were washed in PBS before addition of 0.05–1 mg/ml FGF4 protein in PBS/0.1%BSA (R&D Systems) or 2 mg/ml SU5402 (Calbiochem/CN Biosciences) in DMSO, for 1 h at room temperature.

2.2. Whole-mount in situ hybridisation procedure and probes

Embryos were processed for in situ hybridization as described previously [14]. Sense and antisense *Sulf1* riboprobes (S1–17) were prepared as described previously [4,14]. Sense probe showed no reaction in this

*Corresponding author. Fax: +44 207 468 5204.
E-mail address: tdhoot@rvc.ac.uk (G.K. Dhoot).

0014-5793/$32.00
doi:10.1016/j.febslet.2007.09.033

study or earlier studies [4,14] and therefore was not used in subsequent experiments. The Id2 riboprobe used in this study was the same as described previously (with sense probe showing no reaction) [15]. Msx2 DNA kindly provided by Penny Thomas was linearised with Apa1 and transcribed with T3 RNA polymerase. BMP2, 4 and 7 DNAs were kindly provided by Ketan Patel. BMP2 was linearised with HindIII and transcribed with T3 polymerase. BMP4 was linearised with BamH1 and transcribed with T3 RNA polymerase. BMP7 was linearised with Xho1 and transcribed with T7 RNA polymerase. None of the sense probes showed any staining. Some stained embryos were embedded in 4% agar and 50–100 μm thick sections cut using a vibratome.

2.3. Micromass culture and RT-PCR analysis of limb mesenchymal cells

Limb buds were removed from stage 21–22 embryos and digested in 0.25% trypsin/EDTA at room temperature. The ectoderm was then removed from each limb bud and the mesenchymal cells dispersed by incubation at 37 °C with gentle shaking. Cells were plated as 10 μl spots each containing 5×10^5 cells. Following one day of culture, micromasses were treated with FGF4 (0, 1, 5, 10 ng/ml). Total RNA was harvested from cultures following two days of treatment using Trizol (Invitrogen). For RT-PCR analysis, RNA was reverse transcribed using 3' primers for both *Sulf1* and β-actin. The reaction mixture was then divided into two and PCR performed for either *Sulf1* or β-actin. *Sulf1* primers were 5′-CCTTGTGCTCACAGATGACC-3′ (sense) and 5′-GGCCTATGTGGGTATATCCTC-3′ (antisense) while β-actin was detected using the following primers: 5′-CAATGAGCTGAGAGTAGCCC-3′ (sense) and 5′-GGGTGTTGAAGGTCTCAAAC-3′ (antisense).

3. Results

3.1. FGF4 over-expression in both the interdigit and at the tip of digit III differentially up-regulates Sulf1

At 24 h after implantation of an FGF4 bead (1 mg/ml) into interdigit III, *Sulf1* expression was up-regulated on either side of the bead (Fig. 1A1 and A2). In contrast to this, the expression of BMP2 and BMP4 were slightly reduced around the implanted bead (Fig. 1B1, B2, C1, and C2). At 24 h the interdigital gap between digits III and IV had increased (white dotted bars in Fig. 1B2–D2) and expression of both Id2 and Msx2 in interdigit III also increased (Fig. 1D1 and D2). At 72 h after bead implantation the expression patterns of *Sulf1,* BMP2 and BMP4 were generally similar to controls (Fig. 1A3–C3). However, the morphological changes in the digits were clearly apparent by this time point. With FGF4 beads loaded with 1 mg/ml separation of digits III and IV was normal, however, the persistence of interdigital mesoderm between digits II and III instead (white arrows in Fig. 1A3–D3) suggested a concentration dependent effect on apoptosis. This was further investigated by using beads soaked in different concentrations (0.05–1 mg/ml) of FGF4 that showed a dose dependent persistence of interdigital mesoderm between digits III and IV. At lower doses of 0.05–0.1 mg/ml FGF4 concentration, the effects were minimal (data not shown), however, at doses between 0.25 and 0.5 mg/ml there was retention of tissue within the interdigital region of bead implantation (Fig. 1E).

The implantation of an FGF4 bead at the tip of digit III markedly increased the expression of *Sulf1* at 24 h of re-incubation (Fig. 1F1, F2). *Sulf1* expression in the perichondrium of most normally developing digits (except the distal-most phalange) is restricted to the distal half of the phalange [14]. High levels of *Sulf1* expression following FGF4 bead implantation, however, were observed along the entire perichondrial length of the phalange, encompassing both proximal and distal halves

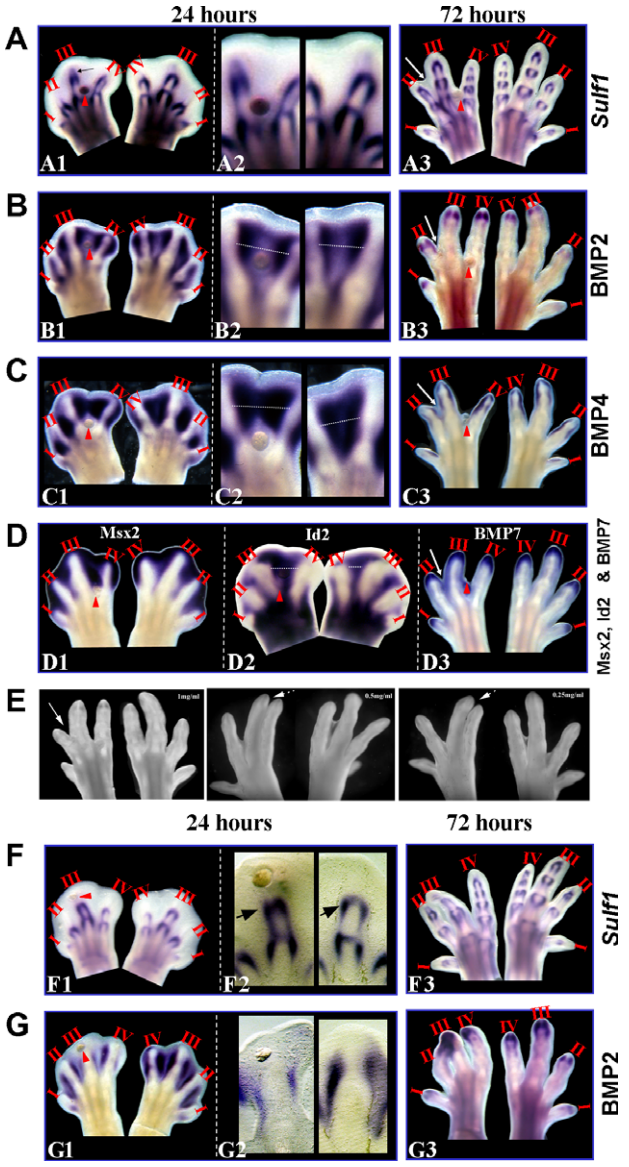

Fig. 1. Expression pattern of *Sulf1*(A), BMP2(B), BMP4(C), Msx2(D1), Id2(D2) and BMP7(D3) after implantation of FGF4-soaked beads (1 mg/ml) in the quail interdigital mesoderm between digits III and IV (A–D) following 24 and 72 h incubation time periods; Syndactyly between digits III and IV 72 h following implantation of FGF beads loaded with 0.25–0.5 mg/ml FGF4 (E); and expression pattern of *Sulf1*(F), BMP2(G) after FGF4 bead implantation at the tip of digit III (D–F) following 24 and 72 h incubation time periods. The identity of the digits is indicated by Roman numerals I, II, III and IV. A2-C2 represent enlarged views of selected regions shown in A1–C1. Red arrow-heads indicate the location of the FGF4 beads. The broken white lines indicate the change in the width of interdigital mesoderm in control versus treated interdigit III. White arrows in Fig. A3–D3 indicate the inhibition of apoptosis between digits II and III instead of digits III and IV. Black arrows in F2 demonstrate increased *Sulf1* expression in distal perichondrium although the spatial pattern in both perichondrium and the joint line remains essentially similar to contralateral control.

(Fig. 1F2). A slightly increased *Sulf1* expression along the lateral sides of such phalanges was still apparent at 72 h, while a slight reduction was observed in the joint line (Fig. 1F3). The implantation of an FGF4 bead at the tip of digit III following 24 h re-incubation showed a reduction in expression of BMP2

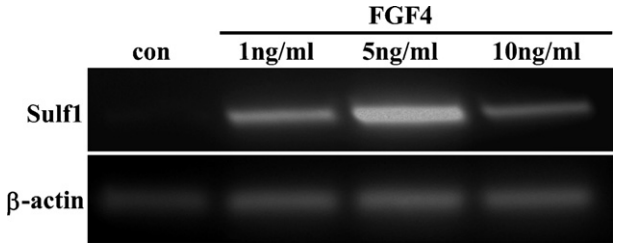

Fig. 2. RT-PCR analysis of *Sulf1* expression in micromass cultures of limb bud mesenchyme treated with FGF4. Cultures were treated with FGF4 (0–10 ng/ml) for two days prior to isolation of total RNA and RT-PCR analysis.

along both sides of digit III but not at a distance along the adjoining lateral sides of digits II or IV (Fig. 1G1, G2). In contrast, BMP2 expression was slightly increased at 72 h (Fig. 1G3). The fusion between digits II and III also became apparent by 72 h from the merging of their BMP2 expression domains (Fig. 1G3). Changes in the expression of BMP4 (not shown) were similar to those seen for BMP2 (Fig. 1G).

3.2. *FGF4 up-regulates Sulf1 expression in cultured micromasses of limb bud mesenchymal cells*

RT-PCR analysis of micromass cultures showed a biphasic dose dependent response of *Sulf1* expression to treatment with FGF4, with expression increasing up to 5 ng/ml and then decreasing at 10 ng/ml (Fig. 2).

3.3. *The over-expression of FGF inhibitor SU5402 in interdigit III down-regulates Sulf1, while its over-expression at the tip of digit III leads to cessation of phalange formation by initially up-regulating Sulf1 at the distal tip*

At 24 h after implantation of an SU5402 bead into the interdigital mesoderm expression of *Sulf1* was down-regulated, particularly in the adjoining lateral sides of both digits III and IV (Fig. 3A1, A2). However, at 72 h the pattern of *Sulf1* expression along the adjoining perichondrial lengths of both digits III and IV was up-regulated, showing a continuous lateral expression and an inhibition of joint formation (Fig. 3A3, A4). Vibratome sectioning of treated autopods confirmed the inhibition of joint formation by SU5402 (Fig. 3A4). The expression of both BMP2 and BMP4 was clearly up-regulated in the interdigit at 24 and 72 h post bead implantation, however, there was little or no effect on BMP7 (Figs. 3B–D). The expression of Id2 at 24 h after implantation of a SU5402 bead was down-regulated in interdigit III, suggesting a lack of apoptosis between digits III and IV (Fig. 3E). This was confirmed at 72 h after bead implantation when autopods showed syndactyly between digits III and IV (Fig. 3A3–E3). Inhibition of the growth of the digits was also apparent from the reduced digital length.

Implantation of an SU5402 bead at the tip of digit III induced a region of ectopic expression of *Sulf1* in the digital tip at 24 h (Fig. 3F1, F2) leading to premature cessation of further phalange formation (Fig. 3F3). Expression of BMP2 was down-regulated slightly at both 24 and 72 h time points. The effect of SU5402 on the expression of BMP4 in the digital tip (data not shown) was similar to that observed for BMP2. At 72 h, treated autopods showed semi-truncated digits and soft tissue syndactyly not only between digits III and IV but also digits II and III (Fig. 3F3, G3).

4. Discussion

Sulf1 has the potential to regulate the activities of FGFs. Thus feedback between FGF signalling and *Sulf1* expression could impact on both chondrogenesis and apoptosis in the developing limb where both are widely expressed and implicated in development and morphogenesis. Over-expression of FGF4 in interdigit III down-regulated *Sulf1* close to the bead but up-regulated expression either side of the bead a short distance away as well as some increase in the adjoining joint line approaching the distal tip. Over-expression of FGF4 at the tip of digit III also resulted in an up-regulation of *Sulf1* expression at 24 h resulting in retarded digital growth. Upregulation of *Sulf1* by FGF signalling was also apparent in micromass cultures of limb bud mesenchyme cells. This up-regulation of *Sulf1* by FGF4 could be acting as part of a negative feedback loop inhibiting FGF signalling by desulfating HSPG co-receptors required for FGF signalling. Although *Sulf1* is expressed at the distal leading edge of completed phalanges, it is specifically excluded from the advancing digital tip until the final phalange is formed [14]. This pattern of expression is compatible with studies showing that attenuation of FGF induces premature tip formation and the hypothesis that the digit tip forms when FGF signalling ceases [16]. The absence of *Sulf1* in the growing digit tip would permit FGF signalling until digital elongation is complete whereupon expression of *Sulf1* in the tip [14] would inhibit FGF signalling resulting in cessation of further phalange formation.

In the developing limb FGFs have been described as inhibiting apoptosis in the short term (12 h), but enhancing apoptosis after 24 h by up-regulating BMP signalling [11]. Our results with implantation of FGF4 beads differ from this showing a dose dependent influence of this FGF. For example, the implantation of a bead soaked in 1 mg/ml FGF4 in interdigit III showed little or no effect on cell death in this interdigit as observed by the normal separation of digits III and IV. It is possible that apoptosis or cell death could have been slightly accelerated in this interdigit but was not apparent from either the 24 or 72 h time intervals investigated in this study. We instead observed a very reproducible inhibitory effect of FGF4 on apoptosis in the adjacent interdigit II. Implantation of FGF4 beads at the tip of digit III also inhibited cell death in interdigit II resulting in the fusion of digits II and III. From these results, we hypothesised that the FGF4 is diffusing to form a gradient and that only at lower concentrations is there an inhibition of apoptosis. We therefore went on to investigate this further by implanting beads loaded with lower concentrations of FGF4 into interdigit III. These experiments confirmed the dose dependence of the effects of FGF4 on apoptosis with low levels of FGF4 (0.05–0.1 mg/ml) having very little effect on apoptosis, in either interdigit II or III, while moderate levels of FGF4 (0.25–0.5 mg/ml) resulted in an inhibition of apoptosis in interdigit III. We therefore conclude that only moderate levels of FGF4 inhibit apoptosis while no effect is observed at high or low concentrations near the implanted bead. Indeed, FGFs have been shown to exhibit divergent concentration dependent effects in other cell systems [17]. Although interdigit II demonstrated inhibition of apoptosis as indicated by the fusion of digits II and III, there was no evidence of significant changes in the expression of BMPs. A slight increase in the expression of Msx2 and Id2 indicates a possible acceleration of apoptosis at high FGF4 concentration in interdigit III.

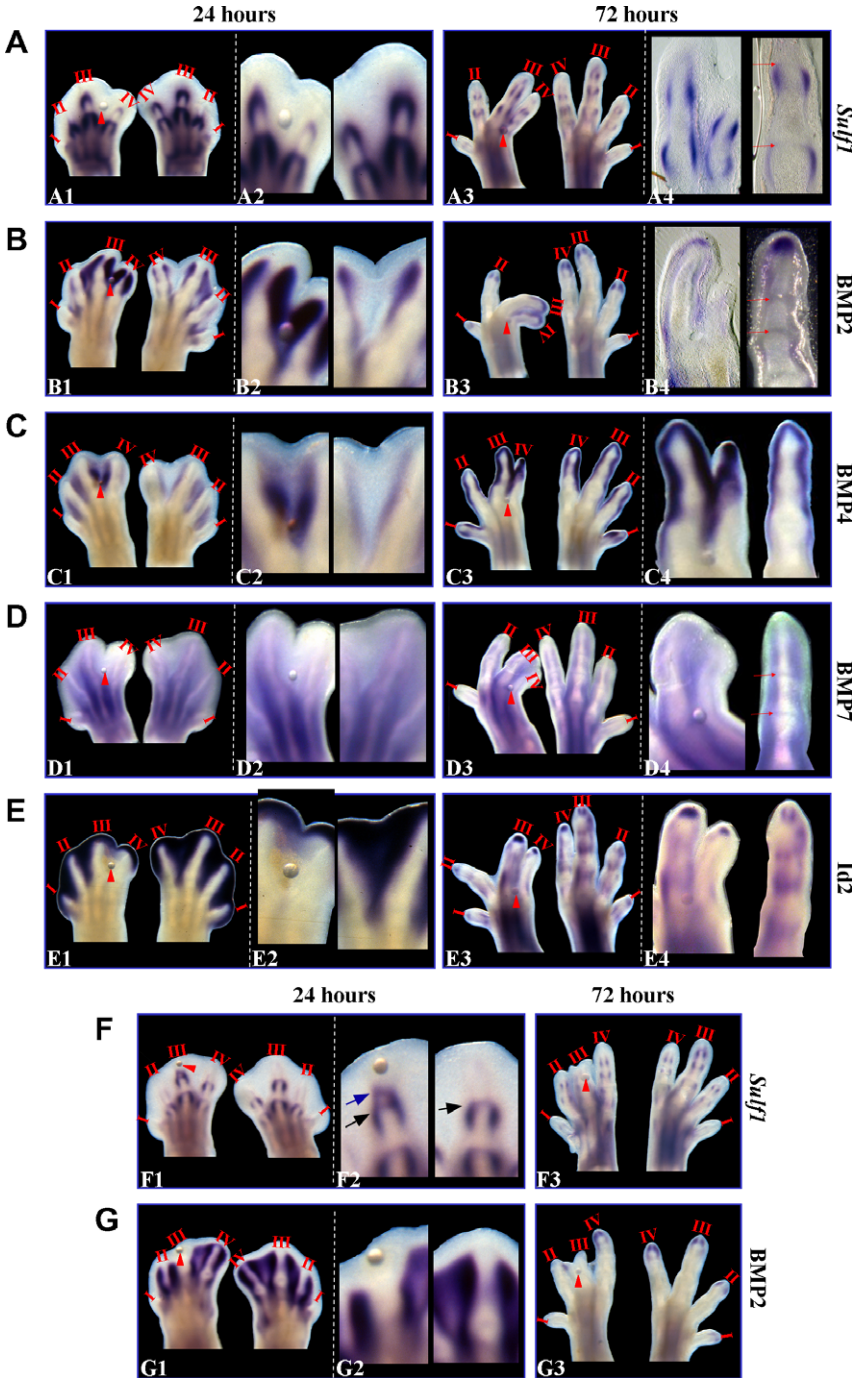

Fig. 3. Expression pattern of *Sulf1* (A), BMP2 (B), BMP4 (C), BMP7 (D) and Id2 (E) after implantation of FGF inhibitor SU5402-soaked beads (2 mg/ml) in the quail interdigital mesoderm between digits III and IV (A–E) following 24 and 72 h incubation time periods; and expression pattern of *Sulf1* (F) and BMP2(G), after SU5402 bead implantation at the tip of digit III following 24 and 72 h incubation time periods. The identity of the digits is indicated by Roman numerals I, II, III and IV. A2–G2 represent enlarged views of selected regions (digits and interdigit III in A1–E1 and digits III in F1 and G1) shown in A1–G1. Red arrow-heads indicate the location of the SU5402 beads. Red arrows indicate the position of the joints in control digits. Black arrows in F2 indicate normal *Sulf1* expression domain in not only the normal but also treated digit III. Digit III treated with SU5402, in contrast shows an additional *Sulf1* expression domain indicated by a blue arrow that is not present in the control. Such an expression pattern characterises terminal digits of all normally developing phalanges [14]. Such induced pattern of *Sulf1* by SU5402 therefore indicates premature termination of digital growth.

Implantation of beads loaded with an FGF inhibitor (SU5402) into interdigital mesoderm initially down-regulated *Sulf1* expression, however, at 72 h the pattern of expression was changed resulting in a continuous line of lateral expression rather than the normal interrupted expression. This expression is most likely perichondrial as our earlier study has shown that this tissue expresses *Sulf1* [14]. Such a sustained expression along the entire length of the perichondrium may inhibit joint formation since *Sulf1* expression in the normally developing digit is interrupted in the lateral regions

near the joints with low level transient expression of *Sulf1* in the joint line [14]. Implantation of an SU5402 bead at the digital tip initially up-regulated *Sulf1* expression and resulted in an inhibition of growth and premature cessation of phalange formation therefore supporting our earlier study showing that over-expression of *Sulf1* terminates further phalange formation [14]. The inhibition of interdigital apoptosis by SU5402, leading to the fusion of digits III and IV, is in agreement with observations by Montero et al. [11]. The fusion, however, was not mediated through down-regulation of BMPs since the levels of both BMP2 and BMP4 were considerably increased. This therefore supports the hypothesis that BMPs cannot induce apoptosis in the interdigit when FGF signalling is blocked [11]. In contrast to this there was a marked decrease in the expression of Id2 in interdigit III, suggesting that Id2 is an important regulator of apoptosis in the developing limb.

While this study shows that implantation of beads loaded with either FGF4 or the inhibitor SU5402 into the tip of digit III resulted in an increase in *Sulf1* expression, it must be noted that there are differences between the expression patterns in these two cases. The *Sulf1* expression pattern in a developing phalange marks the sequential development of each digit [14]. The penultimate digit is characterised by high levels of *Sulf1* expression in distal perichondrium with a lower level in the joint line [14]. The last digit in contrast is characterised by high levels of *Sulf1* expression in the distal end that is believed to mark the cessation of further digit formation as FGFs are inhibited by *Sulf1* [14]. With FGF4 bead implantation at the tip, the spatial *Sulf1* expression pattern is essentially similar to the pattern observed in the contralateral control although the intensity of *Sulf1* expression in the perichondrium had increased considerably (arrows in Fig. 1F2). While FGF4 bead implantation up-regulated *Sulf1* characteristic of a penultimate digit, up-regulation of *Sulf1* by SU5402 in the distal position resulted in an ectopic domain of *Sulf1* expression (blue arrow in Fig. 2F2) resembling the expression pattern of the distalmost part of the digits seen at later stages of development [14]. The ectopic *Sulf1* expression by SU5402 clearly led to premature termination of digit formation resulting in a shortened phalange observed at 72 h. This study therefore demonstrates that the regulation of *Sulf1* expression by FGFs is critical for step-wise phalange generation and joint formation in the limb.

References

[1] Lukatela, G., Krauss, N., Theis, K., Selmer, T., Gieselmann, V., von Figura, K. and Saenger, W. (1998) Crystal structure of human arylsulfatase A: the aldehyde function and the metal ion at the active site suggest a novel mechanism for sulfate ester hydrolysis. Biochemistry 37, 3654–3664.
[2] Knaust, A., Schmidt, B., Dierks, T., von Bulow, R. and von Figura, K. (1998) Residues critical for formylglycine formation and/or catalytic activity of arylsulfatase A. Biochemistry 37, 13941–13946.
[3] Robertson, D.A., Freeman, C., Morris, C.P. and Hopwood, J.J. (1992) A cDNA clone for human glucosamine-6-sulphatase reveals differences between arylsulphatases and non-arylsulphatases. Biochem. J. 288, 539–544.
[4] Dhoot, G.K., Gustafsson, M.K., Ai, X., Sun, W., Standiford, D.M. and Emerson Jr., C.P. (2001) Regulation of Wnt signaling and embryo patterning by an extracellular sulfatase. Science 293, 1663–1666.
[5] Morimoto-Tomita, M., Uchimura, K., Werb, Z., Hemmerich, S. and Rosen, S.D. (2002) Cloning and characterization of two extracellular heparin-degrading endosulfatases in mice and humans. J. Biol. Chem. 2771, 49175–49185.
[6] Ai, X., Do, A.T., Lozynska, O., Kusche-Gullberg, M., Lindahl, U. and Emerson Jr., C.P. (2003) QSulf1 remodels the 6-*O* sulfation states of cell surface heparan sulfate proteoglycans to promote Wnt signaling. J. Cell Biol. 162 (2), 341–351.
[7] Viviano, B.L., Paine-Saunders, S., Gasiunas, N., Gallagher, J. and Saunders, S. (2004) Domain-specific modification of heparan sulfate by Qsulf1 modulates the binding of the bone morphogenetic protein antagonist Noggin. J. Biol. Chem. 279, 5604–5611.
[8] Lai, J., Chien, J., Staub, J., Avula, R., Greene, E., Matthews, T., Smith, D., Kaufmann, S., Roberts, L.R. and Shridhar, V. (2003) Loss of HSulf-1 up-regulates heparin-binding growth factor signaling in cancer. J. Biol. Chem. 278, 23107–23117.
[9] Wang, S., Ai, X., Freeman, S.D., Pownall, M.E., Lu, Q., Kessler, D.S. and Emerson Jr., C.P. (2004) QSulf1, a heparan sulfate 6-*O*-endosulfatase, inhibits fibroblast growth factor signaling in mesoderm induction and angiogenesis. Proc. Natl. Acad. Sci. 101, 4833–4838.
[10] Martin, G.R. (1998) The roles of FGFs in the early development of vertebrate limbs. Genes Dev. 12, 1571–1586.
[11] Montero, J., Ganan, Y., Macias, D., Rodriguez-Leon, J., Sanz-Ezquerro, J., Merino, R., Chimal-Monroy, J., Nieto, M.A. and Hurle, J.M. (2001) Role of FGFs in the control of programmed cell death during limb development. Development 128, 2075–2084.
[12] Yamaguchi, T. and Rossant, J. (1995) Fibroblast growth factors in mammalian development. Curr. Opin. Genet. Dev. 5, 485–491.
[13] Ngo-Muller, V. and Muneoka, K. (2000) Influence of FGF4 on digit morphogenesis during limb development in the mouse. Dev. Biol. 219, 224–236.
[14] Zhao, W., Sala-Newby, G. and Dhoot, G.K. (2006) Expression pattern of *Sulf1* and its role in cartilage and joint development. Dev. Dyn. 235, 3327–3335.
[15] Krishan, K., McKinnell, I., Patel, K. and Dhoot, G.K. (2005) Dynamic Id2 expression in the medial and lateral domains of avian dermamyotome. Dev. Dyn. 234, 363–370.
[16] Sanz-Ezquerro, J.J. and Tickle, C. (2003) Fgf signaling controls the number of phalanges and tip formation in developing digits. Curr. Biol. 13 (20), 1830–1836.
[17] Garcia-Maya, M., Anderson, A., Kendal, C., Kenny, A., Edwards-Ingram, L., Holladay, A. and Saffell, J.L. (2006) Ligand concentration is a driver of divergent signaling and pleiotropic cellular responses to FGF. J. Cell Physiol. 206, 386–393.