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Growth factors FGF8 and FGF2 and their receptor FGFR1, transcriptional factors Msx-1 and MSX-2, and apoptotic factors p19 and RIP5 participate in the early human limb development

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ABSTRACT

The expression pattern of fibroblast growth factors FGF8 and FGF2 and their receptor FGFR1, transcription factors MSX-1 and MSX-2, as well as cell proliferation (Ki-67) and cell death associated caspase-3, p19 and RIP5 factors were analyzed in histological sections of eight 4th-9th-weeks developing human limbs by immunohistochemistry and semi-thin sectioning. Increasing expression of all analyzed factors (except FGF8) characterized both the multilayered human apical ectodermal ridge (AER), sub-ridge mesenchyme (progress zone) and chondrocytes in developing human limbs. While cytoplasmic co-expression of MSX-1 and MSX-2 was observed in both limb epithelium and mesenchyme, p19 displayed strong cytoplasmic expression in non-proliferating cells. Nuclear expression of Ki-67 proliferating cells, and partly of MSX-1 and MSX-2 was detected in the whole limb primordium. Strong expression of factors p19 and RIP5, both in the AER and mesenchyme of human developing limbs indicates their possible involvement in control of cell senescence and cell death. In contrast to animal studies, expression of FGFR1 in the surface ectoderm and p19 in the whole limb primordium might reflect interspecies differences in limb morphology. Expression of FGF2 and downstream RIP5 gene, and transcription factors Msx-1 and MSX-2 did not show human-specific changes in expression pattern. Based on their spatio-temporal expression during human limb development, our study indicates role of FGFs and Msx genes in stimulation of cell proliferation, limb outgrowth, digit elongation and separation, and additionally MSX-2 in control of vasculogenesis. The cascade of orchestrated gene expressions, including the analyzed developmental factors, jointly contribute to the complex human limb development.

1. Introduction

In the early human development, limb buds can be observed as outpocketings of the ventrolateral body wall, which during growth, progress through the mesenchymal, chondrogenic and osseous developmental phases. During the earliest stages of limb formation, two important signaling centers, the epithelial apical ectodermal ridge (AER) and the mesenchymal zone of polarizing activity (ZPA), are formed and they mutually interact thus regulating the processes of cell proliferation, cell death and limb outgrowth (Kuhlman and Niswander, 1997). Histologically, AER can be described as the ectodermal thickening covering the outer surface at the distal tip of the limb bud, which in accordance to its role as a signaling center expresses a number of different genes and consequently undergoes numerous morphogenetic changes (Fernandez-Teran and Ros, 2008). It should be noted, though, that there are some significant inter-species differences with regard to both morphology of the AER and its overall signaling activity in developing limbs. For example, while in chick embryos AER is present as a pseudostratified epithelium, in mouse embryos AER is of polystratified epithelium type (Bell et al., 1998). It is also believed that human AER is more similar to the AER of mouse (Boulet et al., 2004). Furthermore, in contrast to tetrapod limb development, AER-derived signals are completely shut off in fins, which might be a sign of evolutionary adaptation (Masselink et al., 2016). Among numerous factors involved in the control of proper limb development and patterning, members of the fibroblast growth factor (FGF) superfamily have been shown to have a very important role (Boulet et al., 2004), which is executed by their binding to receptors of tyrosine kinase family (FGFRs) (Celli et al.,

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1998). Gene knockout experiments have positioned the *fgfr1* gene at the top of the regulatory gene hierarchy, which by affecting downstream genes controls cell proliferation and differentiation in developing limb buds (Stachowiak and Stachowiak, 2016).

Two particular members of the FGF family, i.e. FGF2 and FGF8, were shown to have crucial influence on the earliest stages of limb development as they are produced in the AER region and function as signals that promote proliferation and survival of the underlying mesenchyme in the progress zone of distal structures (Hernandez-Martinez et al., 2009). Generally, FGF2 seems to be a wide-spectrum mitogenic, angiogenic, and neurotrophic factor that is expressed in many developing tissues and cell types, including the developing limb buds, which was best demonstrated in experiments where beads soaked in FGF2 were able to partially substitute the signaling activity of AER since proliferation of limb bud mesenchyme was maintained in spite of the absence of AER, while in limbless chickenonly FGF-2 protein was present in the limb bud ectoderm (Ros et al., 1996). Similarly, FGF8 serves as an endogenous inducer of chick limb formation, in a way that it triggers and sustains limb development, while acting synergistically with certain morphogens (such as IHH), which is especially important for promotion of chondrogenesis during digit primordia elongation (Zhou et al., 2007). Experiments that included application of FGF beads to the flank, stimulated mesenchyme proliferation in the limb region and the formation of an ectopic ridge leading to an ectopic limb formation (Cohn et al., 1995; Kuhlman and Niswander, 1997). In contrast, application of FGFR inhibitor was shown to lead to a reduced number of phalanges.

One of the downstream genes in FGF signaling pathway is RIP5 (DSTYK), a member of serine/threonine kinase family involved in induction of apoptosis. RIP5 is expressed in the cell membranes of maturing epithelia of many organs, and co-localizes with FGFRs in the ureteric bud and metanephric mesenchyme, thus making it a major determinant of human urinary tract development (Sanna-Cherchi et al., 2013). In RIP5 mutants, multiple organs are affected including limbs, and that phenotype can partly be explained by improper FGFR signaling, which is essential for maintaining inductive interactions and outgrowth of limb primordia. Similarly, in *Fgfr1* mouse mutants the digit number and identity are affected (Verheyden et al., 2005). It should be noted, however, that conditional deletion of *Fgfr1* in the early stages of limb bud development results in more severe limb defects by triggering cells death and alterations of genes related to apoptosis, which significantly affects the advanced stages of limb formation as well (Li et al., 2005). In addition, altered expression of genes encoding transcription factors Msx-1 and Msx-2 also characterizes aberrant FGFR signaling (Celli et al., 1998). During the early development, Msx-1 and Msx-2 are normally expressed in various tissues harboring progenitor cell populations, and are generally associated with maintenance of proliferation potential. Thus, in limb primordia *Msx-1* and *Msx-2* genes were shown to be highly expressed in the limb field from the earliest stages of limb formation in both the apical ectodermal ridge and underlying mesenchyme, while their miss-expression induced formation of ectopic AERs. *Msxs* seem to act as downstream genes in the signaling cascade of bone morphogenesis protein (BMP) as well. Also, *Msx-1* and *MSX-2* are expressed in the interdigital tissue at the time when apoptosis takes place (Lallemand et al., 2005). The regenerative potential of digital tips has been correlated with the expression domain of *Msx-1* gene in that region (Reginelli et al., 1995). In addition, both genes have specific roles in craniofacial and tooth development (Alappat et al., 2003; Kero et al., 2016; Kero et al., 2017).

Fine balance between factors controlling cell proliferation and apoptosis is essential for normal limb formation. Among them, p19, which belongs to a family of INK4 cyclin-dependent kinase inhibitors (CKI), is important for inducing terminal differentiation of cells by blocking the progression of cell cycle during the G1 phase. p19 is, thus, involved in senescence, apoptosis, DNA repair, and in multistep oncogenesis (Canepa et al., 2007). Knockdown of p19 renders cells sensitive to apoptotic and autophagic types of cell death (Tavera-Mendoza et al., 2006), but so far its role in regulation of limb development, to the best of our knowledge, has not been investigated. However, recent studies on human limb development indicate that both caspase-3 and AIF–dependent pathways of cell death might be involved in AER formation and in control of cell numbers in progress zone and zone of digital separation. This implies that p19 might also have distinct role in the regulation of the aforementioned processes in developing limb. Furthermore, investigation of p19 expression patterns might also shed some light on regulatory background of the spatiotemporal changes in proliferation during limb outgrowth and digit separation, which we had previously analyzed by application of Ki-67 marker (Becic et al., 2016), a nuclear protein present in all phases of the cell cycle, except the G0 phase (Key et al., 1994).

The process of limb formation is strikingly complex, but genetic interactions and signaling pathways are still not fully understood, particularly in human development. Most of the data on genetic control of limb development are coming from experimental studies, showing interspecies differences in limb patterning inspite the fact that basic mechanisms of limb development have been evolutionary highly conserved. In the present study we analyze growth factors and their receptors, as well as downstream genes involved in control of the early stages of human limb development in order to indetify possible specificities of human limb development.

2. Materials and methods

2.1. Tissue procurement and processing

All embryonic and fetal tissues were collected and processed with the permission of the Ethical and Drug Committee of the University Hospital of Split, in accordance with the Helsinki Declaration (Williams, 2008). After external examination of the conceptuses, any macerated or poorly preserved material was discarded. The age of conceptuses was estimated according to external measurements (crown–rump length) and the Carnegie staging system based on morphological appearance (O'Rahilly, 1972). A total of eight normal human conceptuses between 4th and 10th developmental weeks were taken from the collection of human tissues in the Department of Anatomy, Histology and Embryology, School of Medicine University of Split (collected between 2012 and 2017). The tissue was fixed in 4% paraformaldehyde in phosphate buffer saline (PBS), dehydrated in graded ethanol and paraffin-embedded. Serial 7 μm thick sections were cut in transversal or longitudinal plane, mounted on aminopropyl triethoxysilane-treated glass slides, and analyzed using an Olympus BX51 light microscope (Olympus, Tokyo, Japan). Haematoxylin and eosin staining of every 10th section of each tissue block confirmed appropriate tissue preservation.

2.2. Immunohistochemical and immunofluorescence staining

After deparaffinization and rehydration, the sections were treated as previously described (Kalibović Govorko et al., 2010; Tafra et al., 2014). Sections were cooked in sodium citrate buffer for 17 min at 95 °C. After being cooled to room temperature, slides were incubated with the following primary antibodies (separately or in appropriate combination): rabbit anti-human/mouse active caspase-3 (1:1500; AF835, R&D Systems, Minneapolis, MN, USA), goat monoclonal anti-human MSX-1 antibody (1:300; ab93287, Abcam, UK), mouse monoclonal Anti-FGFR1 antibody (1:300; ab824, Abcam, UK), rabbit polyclonal anti-human Anti-MSX-2/Hox8 antibody (1:300; ab190070, Abcam, UK), rabbit polyclonal anti-human Anti-p19 INK4d antibody (1:100; ab102842, Abcam, UK), mouse monoclonal anti-human Ki-67 antibody (1:50; DAKO, Glostrup, Denmark), rabbit polyclonal anti-human FGF2 antibody (1:500; ab8880, Abcam, UK), rabbit polyclonal Anti-RIPK5 antibody (1:500; ab153997, Abcam, UK) and goat