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Skin and scale regeneration after mechanical damage in a teleost

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ABSTRACT

Skin wound healing has been widely studied in mammalian models but the information on teleost cutaneous healing is sparse and frequently considered in the context of viral or bacterial infections or parasitic infestations in aquaculture. In the present study a detailed time course (0 h, 6 h, 1, 2, 3 and 4 days) coupled to morphology and gene expression analysis revealed rapid regeneration of skin without scarring in a marine teleost after a superficial wound caused by the loss of a large area of scales. The integrity of the integument, as assessed by quantification of extracellular matrix (ECM) gene transcripts (*fn1a*, *collα1*, *colVα2*, *colXα1*, *ogn1*, *ogn2*, *crtac1a*, *cyr61*, *pcna*, *krt2* and *mmp9*) was restored within 2 days. Epithelial-mesenchyme interactions assessed by expression of *edar* and *shh* were associated with epidermal closure, the re-establishment of the basement membrane and also scale eruption. Histological observations suggested tissue re-epithelialization was independent of inflammation and that transcripts representing the humoral and cellular elements of the immune response (*mpo*, *cyba* and *csf1r*, *cd48* and *cd200*) were modulated in the early stages of sea bream (*Sparus aurata*) skin repair after injury. Overall, the results indicate that after superficial skin damage tissue reconstitution started immediately with re-epithelialization, followed by ECM deposition and finally tissue maturation, indicating that in the skin regenerative process, reconstitution of the physical barrier was the priority over other integument functions, including immune surveillance.

1. Introduction

Fish skin is rich in mucous-producing cells, lacks keratinization and is composed of living epithelial cells that are in direct contact with the external aquatic environment and when damaged are rapidly repaired (Gomez et al., 2013; Salinas et al., 2011) to re-establish the broken physical barrier and its protective immune functions. The sequence of events that leads to repair or regeneration in tetrapods (Godwin et al., 2014; Godwin and Rosenthal, 2014; Olczyk et al., 2014; Yates et al., 2012; Midwood et al., 2004) and teleosts (Richardson et al., 2013; Rai et al., 2012; Guerra et al., 2008), occurs through similar biological processes that overlap in time and space to restore tissue integrity and lead to different outcomes such as the formation of a scar or tissue regeneration (Seifert and Maden 2014; Seifert et al., 2012b,a; Gomez et al., 2013). In both animal groups the main stages of wound repair are the inflammatory phase, followed by re-epithelialization and new tissue formation and remodelling (Olczyk et al., 2014; Richardson et al., 2013; Ángeles Esteban, 2012; Eming et al., 2009). In teleosts, the immune system has a key role during regeneration since immune cells destroy and remove pathogens, damaged cells and extracellular structures, and this process is an essential prerequisite for the onset of tissue repair (Midwood et al., 2004).

There are relatively few studies of cutaneous wound healing in fish but based on the studies available it is clear that the severity of wounding affects the repair program. When damage is superficial and affects mainly the epidermis and loose dermis, such as occurs during scale loss, skin repair is rapid. For example, re-epithelialization and differentiation of scale-forming cells is completed within 1–2 days after scale removal in medaka (*Oryzias latipes*), sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus aurata*), by 3–5 days the bony matrix of

Abbreviations: ECM, extracellular matrix; *fn1a*, fibronectin 1a; *collα1*, collagen type I, alpha 1; *colVα2*, collagen type V, alpha 2; *colXα1*, collagen type X, alpha 1; *ogn1*, osteoglycin 1; *ogn2*, osteoglycin 2; *crtac1a*, cartilage acidic protein 1a; *cyr61*, cysteine-rich angiogenic inducer; *pcna*, proliferating-cell nuclear antigen; *krt2*, keratin 2; *mmp9*, matrix metalloproteinase; *edar*, ectodysplasin a receptor; *shh*, sonic-hedgehog; *mpo*, myeloperoxidase; *cyba*, cytochrome b-245; *csf1r*, macrophage colony-stimulating factor 1 receptor; *cd48*, cd48 antigen; *cd200*, ox-2 membrane glycoprotein; RT-qPCR, real-time quantitative polymerase chain reaction; EDTA, ethylenediaminetetraacetic acid; APES, 3-aminopropyltriethoxysilane; PFA, paraformaldehyde; cDNA, complementary deoxyribonucleic acid; bp, base pairs; *scpp1*, Secretory Calcium-binding Phosphoprotein 1; *scpp5*, Secretory Calcium-binding Phosphoprotein 5; hpw, hours post wounding; dpw, days post wounding; *tgfb*, transforming growth factor beta

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the scale is produced, then at 6–14 days the scale basal-plate matrix is produced, followed by scale calcification at days 14–28 (Ohira et al., 2007; Guerreiro et al., 2013; Costa et al., 2017). When wounding is more severe and is associated with bleeding, in teleosts cutaneous wound healing starts immediately and unlike mammals is independent of signals released from the blood clot (Richardson et al., 2013).

Several recent studies have targeted fish skin due to the impact on aquaculture production of the loss of skin integrity due to damage (Rakers et al., 2010; Ceballos-Francisco et al., 2017; Verma et al., 2017; Cordero et al., 2017b). In a microarray study of sea bream (*Sparus aurata*) in which fish were fasted and the skin was damaged by scale removal, the modified processes inferred from up-regulated genes at 3 and 7 days after damage were immune surveillance, tissue regeneration and mitotic checkpoint and cell proliferation (Vieira et al., 2011). Recent studies in the sea bream, have revealed that the thickness of the epidermis, epithelial cell area and area occupied by microridges and immunoglobulin T (Barclay et al., 2002) expression are highest in dorsal skin (Ceballos-Francisco et al., 2017; Cordero et al., 2017b). Furthermore, the mucous composition of wounded sea bream skin correlates with increased susceptibility for infections (Cordero et al., 2017a) and chronic stress impairs the local immune response during cutaneous repair (Mateus et al., 2017). A microarray study in sea lice (*Lepeophtheirus salmonis*) infested Atlantic salmon (*Salmo salar*) revealed a mixed inflammatory response and delayed wound healing (Krasnov et al., 2012; Skugor et al., 2008) but the increase of cortisol levels were found to have a greater impact on the fish immune and healing response rather than parasite infestation alone (Krasnov et al., 2012).

The time dependent dynamics of the immune response means that deciphering fish skin regeneration/repair requires detailed studies across time of the ongoing molecular, cellular and organ specific processes. In the present study, we characterised for the first time in a teleost, a detailed time course of skin repair after extensive surface damage caused by scale removal. Matched samples of damaged and undamaged tissue from the same donor fish differentiated local damage specific events from systemic effects. To capture early and delayed responses to damage, a detailed chronology (0 h, 6 h, 1, 2, 3 and 4 days) of the main biological events associated with skin regeneration, starting immediately after wounding was mapped using the pattern of gene expression and histological observations.

2. Materials and methods

2.1. Selection of candidate genes

Eighteen candidate genes, involved in biological processes of interest (Table 1), were selected from: i) transcripts that were significantly modified in a previous microarray study (Vieira et al., 2011 and Supplementary Table S1 (Conesa et al., 2005; Altschul et al., 1990)), ii) differentially expressed proteins reported during sea bream skin repair (Ibarz et al., 2013) or iii) genes identified from bibliographic searches. A comprehensive description of the molecular profile of candidate genes across a detailed time course (0 h, 6 h, 1, 2, 3 and 4 days) was established by real time-quantitative PCR (RT-qPCR) during skin regeneration in sea bream.

2.2. Skin regeneration challenge

The maintenance of fish and experiments complied with the Guidelines of the European Union Council (86/609/EU) and were covered by a group 1 license from the Portuguese Government Central Veterinary service to CCMAR and conducted by a certified investigator (DMP). The behaviour and health of animals was monitored visually each day and no evidence of infection, modified behaviour or mortality occurred during the experiment.

2.2.1. Fish

A stock of adult sea bream (*Sparus aurata*) of the same age class (1-year-old) were purchased from a commercial supplier (CUPIMAR SA, Cádiz, Spain) and transferred to Ramalhete the experimental station of the Centre of Marine Sciences, University of Algarve (Faro, Portugal). Fish were acclimated to 1000 L tanks supplied with a continuous flow of aerated sea water at 18–20 °C, pH 7.8–8.1, 37 ppt salinity, > 80% oxygen saturation and at a density of < 5 kg m^{-3}. For maintenance fish were fed *ad libitum* twice daily with a commercial feed (Excel; Skretting, Burgos, Spain).

2.2.2. Skin regeneration experiment

For the skin regeneration challenge adult sea bream (n = 40, length = 34 ± 1.3 cm) were randomly divided between five 500 L tanks (n = 6 per tank) supplied with a continuous flow of aerated

Table 1
Candidate transcripts selected for expression analysis in damaged sea bream skin.

Biological process	Gene	Gene name	Function
Re-epithelialization	*pcna*	*proliferating-cell nuclear antigen*	Marker of proliferation during wound healing (Braiman-Wiksman et al., 2007)
	krt2	*keratin 2*	Type II keratin expressed in fish skin (Infante et al., 2007)
	mmp9	*matrix metalloproteinase 9*	Tissue degradation and removal of cellular debris (Gawronska-Kozak 2011)
ECM and matricellular protein deposition	*fn1a*	*fibronectin 1a*	Ubiquitous cell adhesive ECM protein involved in wound repair (Midwood et al., 2004)
	colVa2	*collagen type V*	Minor collagen in fish skin and scales (Guellec and Zylberberg 1998)
	cyr61	*cysteine-rich angiogenic inducer 61*	Inducer of angiogenesis (Chen et al., 2001)
	edar	*ectodysplasin a receptor*	Development of integumentary appendages (Harris et al., 2008; Cui and Schlessinger 2006)
	shh	*sonic-hedgehog*	Important roles in organogenesis, including epithelial-mesenchymal interactions (Sire and Akimenko 2004)
Tissue maturation/scale mineralization	*colIa1*	*collagen type I*	Major protein component of cartilage, bone and skin
	colXa1	*collagen type X*	Marker of chondrogenesis in sea bream (Estevao et al., 2011)
	ogn1	*osteoglycin 1*	Regulates collagen fibrillogenesis (Tasheva et al., 2002)
	ogn2	*osteoglycin 2*	Unknown function in teleost
	crtac1a	*cartilage acidic protein 1a*	Promotes piscine epithelial cell outgrowth *in vitro* (Anjos et al., 2013, Anjos et al., 2017)
Immunity	*mpo*	*myeloperoxydase*	Marker of leukocyte activation in sea bream (Rodriguez et al., 2003)
	csf1r	*colony-stimulating factor receptor 1*	Macrophage marker in sea bream (Roca et al., 2006)
	cyba	*cytochrome b-245*	Antimicrobial response of primary phagocytes (neutrophils) (Grayfer et al., 2011)
	cd48	*cd48 antigen*	Surface antigen in mammalian lymphocytes but with undescribed function in teleost (Sameshima et al., 2012)
	cd200	*ox-2 membrane glycoprotein*	Differentiation of myeloid cells (Barclay et al., 2002); immunosuppressive molecule biomarker of hair follicle bulge in human and dog skin (Gorczynski et al., 2010, Jiang et al., 2010, Kobayashi et al., 2010)