Melanosome transfer, photoreception and toxicity assays in melanophores

Daniel Hedberg

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DISSERTATION ABSTRACT

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Many animals such as fish and frogs have developed the ability to change colour of their skin to adapt to the environment or to signal to other individuals. This ability is due to specialised skin cells called melanophores. Melanophores contain thousands of melanosomes, small membrane-enclosed organelles containing the black or brown pigment melanin. The melanosomes can aggregate to the cell centre rendering the cells pale or disperse throughout the cell to become dark. The intracellular transport of melanosomes is regulated by neuronal or hormonal external stimuli. Fast colour change is achieved by aggregation/dispersion of melanosomes but long-term colour change can also be achieved by melanosome transfer to surrounding skin cells.

An amphibian immortalized melanophore cell line was used from the African claw frog, *Xenopus laevis* to study transfer of melanosomes to co-cultured fibroblasts. Melanosome transfer was observed and up regulated by the hormone α-MSH . The transfer was quantified using light-, fluorescence and electron microscopy.

A new and powerful method for transfer experiments was developed. Fluorescent semiconductor nanocrystals, qdots, were used in combination with flow cytometry. The qdots were taken up by the cultured *Xenopus laevis* melanophores, localised to the melanosomes and transferred to co-cultured fibroblasts. The method is a step towards enabling large scale analysis of pigment transfer.

Xenopus laevis melanophores can be cultivated in 96-well culture plates which allow quantification of aggregation or dispersion in a fast and reproductive way. Glyphosate containing herbicides, i.e. Roundup, are commonly used in the world, but some toxic effects have been found on amphibians *in vivo* and human and mouse cells *in vitro*. To learn more about potential effects on intracellular transport and the cytoskeleton in animal Roundup, glyphosate, glyphosateisopropylamine and isopropylamine were tested on the transport of melanosomes to the cell centre by spectrophotmetry and by fluorescence microscopy on microtubules and actin filaments. All tested compounds inhibited the aggregation and affected the morphology of the cytoskeleton. The effect was found to be pH dependent.

Amphibian melanophores can be regulated directly by light via a melanopsin receptor. Photoreception was found in cultured early embryos of the zebrafish *Danio rerio.* Light was found to induce dispersion of the melanophores. In adults light causes aggregation of the melanosomes due to signals from the CNS. At least one subclass of melanopsin was detected in the zebrafish retinal pigment epithelial cells.

Key words: colour change, melanophore, melanosome, intracellular transport, pigment transfer, photoreception, toxicity assay, Roundup, glyphosate

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LIST OF PAPERS

The thesis is based on the following paper and manuscripts, which are referred to in the text by their Roman numerals:

I Aspengren, S., Hedberg, D., and Wallin, M. Studies of pigment transfer between *Xenopus laevis* melanophores and fibroblasts in vitro and in vivo. Pigment Cell Research, 2006, 63, 423-436.

II Hedberg, D., Wetterskog, D., and Wallin, M. Fluorescent semiconductor nanocrystals, qdots, as a novel tool in studies of melanosome transfer. *In manuscript.*

III Hedberg, D., and Wallin, M. Effects of Roundup and glyphosate formulations on intracellular transport, microtubules and actin filaments in *Xenopus laevis* melanophores. *Under revision for publication.*

IV Hedberg, D., Gräns, J., and Wallin, M. Photoreception and signal transduction in zebrafish melanophores. *In manuscript.*

ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
ATP	Adenosine 5 triphosphate
CaM	Calmodulin
cAMP	cyclic adenosine monophosphate
CFDA/CMFDA	5-Chloromethylfluorescein diacetate
DAG	Diacylglycerol
DCT	Dopachrome tautomerase
DiI	1,1´-dioctadecyl-3,3,3´,3´-tetramethylindocarbocyanine perchlorate
DiO	3,3`-dioctadecyloxacarbocyanine perchlorate
DMEM	Dulbecco's modified Eagle medium
DOPA	L-3,4-dihydroxiphenylalanine
EGFP	Enhanced green fluorescent protein
EM	Electron microscopy
EP1 receptors	E-prostaglandin receptor 1
EP3 receptors	E-prostaglandin receptor 3
EPSPS	5-enolpyruvyl-shikimate-3-phosphate
ER	Endoplasmic reticulum
ET-1	Endothelin-1
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
FITC	Fluorescein isohiocyanate
FSC	Forward scattering
G-proteins	GTP binding proteins
GFP	Green fluorescent protein
GPCR	G Protein coupled receptors
GTP	Guanosine 5 triphosphate
HPS	Hermansky-Pudlak syndrome
ILV	Intraluminal vesicles
IP_3	Inositol-1,4,5-triphosphate
ipRGC	intrinsically photosensitive retinal ganglion cells
MAP	Microtubule-associated proteins
MC1 Receptor	Melanocortin 1 receptor
MITF	Microphtalmia-associated transcription factor
α-MSH	α-Melanocyte-stimulating hormone
MT	Microtubules
NO	Nitric oxide
PAR 2	Protease activated receptor 2
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
PE	Phycoerythrin
PIP2	Phosphatidylinositol 4,5 biphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PMA	Phorbol myristate acetate
PP2B	Protein phosphatase 2B
Qdot	Quantum dot; fluorescent semiconductant nanocrystal
RGS	Regulators of G protein signalling
RILP	Rab-Interacting lysosomal protein
RNAi	RNA interference
ROS	Reactive oxygen species
RPE	Retinal pigment epithelial
RPMI	Roswell park memorial institute medium
RT-PCR	Real time polymerase chain reaction
SNARE	Soluble N-ethylmaleimide sensitive factor
SSC	Side scattering
TPA	12-0-tetradecanoyl phorbol acetate
TRP1	Tyrosinase related protein 1
UV	Ultraviolet light
XB-2	A teratocarcinoma derived cell line of keratinocyte lineage

INTRODUCTION

The nature of pigment cells

Throughout the animal kingdom adaptation of skin colour is an essential factor for survival of the individual and the success of the species. In the daily strife to survive it is important to avoid detection from predators and potential preys, not becoming too warm or too cold from exposure or the lack of exposure from the sun. The sun will not only warm the skin, but at the same time damage the DNA and induce production of beneficial factors such as vitamin D. If walking this tightrope is not enough, the skin colour must also function as a device for communication within species to reflect mood, individuality, social position and dominance, gender and sexual readiness, health status and fitness. At the same time it may be practical in some cases to signal to other species in an effort to remind predators that the animal is poisonous, or at least mimic someone who is.

While humans have devised technological solutions, i.e. clothes, to increase the ability to adapt to both environmental stresses and ability for signalling in a social context, the majority of life on earth must survive and adapt by using only one set of clothes; their skin.

As a single skin colour rarely would meet all listed requirements at the simultaneously, ingenious solutions has been derived during evolution to accommodate the needs in different species, individuals and body parts. One such solution is the use of pigmented skin cells generally termed chromatophores. The chromatophores can be divided into subtypes depending on the colour of the pigment in the cell; melanophores (brown-black), xantophores (yellow), erythrophores (red), and leucophores/iridophores (white-metal). In general, chromatophores have mostly been studied in fish and amphibians and the cell type most extensively studied is the dark pigmented melanophore. The black colour arises from production of the dark pigment melanin in specialized organelles, termed melanosomes, within the melanophores. The mammalian equivalent of the melanophore is called melanocyte and though they share common origins they differ slightly in localization, regulation and functionality.

This thesis will mainly address biological processes in fish and amphibian melanophores, and will to some extent discuss the mammalian melanocyte as there is a remarkable and interesting loss and gain of functions during evolution of this cell type .

Melanophores and melanocytes

The remarkable patterning and range of beautiful colouration in fish is by large the result of the distribution of chromatophores in the dermis and scales. One example is the striped patterning in zebrafish, *Danio rerio*, where the stripes are composed of melanophores in the dark stripe, and yellow xantophores and light reflecting irridophores in the yellow stripes. All cell types reside in layers in the hypodermis (Hirata et al., 2003). Chromatophores are also present in the scales and dorsal scales display a higher density of melanophores compared to ventral scales with a gradient in between thus creating the appearance of the dark back and the pale belly. The patterning and brightness of the animal is by large a result of the density of a given chromatophore in a given area. In fact, one way of colour adaptation for fish is to increase or decrease the number of melanophores in the skin via cell division or apoptosis (Sugimoto et al., 2002, 2005). This kind of adaptation is relatively slow, it takes place during several weeks and is often termed morphological colour change. In contrast, rapid and reversible colour change is possible by regulating the distribution of the dark melanosomes within each melanophore. This is termed physiological colour change and is a process which can have dramatic results. An area of the skin can be completely covered with melanophores and appear black when the melanosomes are evenly dispersed within each cell. In response to external signals, the melanophores are able to rapidly aggregate their melanosomes to the cell centre. When melanophores cover other chromatophores in the skin they will hide them during dispersion, and reveal them during aggregation. These chromatophore units can be complex and further modification of the pigment in the chromatophores can enhance this effect**.**

Chromatophore units are also present in many amphibians. In the African clawed frog, *Xenopus laevis*, the skin is composed of the epidermis, a basal lamina and the dermis. The dermis can be further divided into the *stratum spungiosum*, containing large glands secreting mucus to the surface of the skin, and the inner *stratum compactum*, containing fibroblasts and connective tissue. Melanophores are present in all layers of the skin; they line the inner surface and blood vessels in the *stratum compactum*, and they line the secretory glands in *stratum spungiosum*. Large melanophores are present under the basal lamina in addition to yellow xantophores and light-reflecting irridophores to compose a primitive chromatophore unit, whereas smaller melanophores resides in the epidermis. As many amphibians, *Xenopus* appears to have green colours in some areas even if there are no known chromatophores with green pigment. A hypothetical explanation of this phenomenon (Bagnara and Hadley, 1973) is that incoming light is filtered through the xantophore layer, reflected and scattered on the underlying irridophore and melanophore layer and then re-filtered through the xantophore layers. The importance of xantophores for green colour formation is illustrated further by experiments in *P. dacnicolor* where individuals who lack functional xantophores either by mutation or malnutrition will turn blue (Bagnara and Matsumoto 2006).

The epidermal melanophores have the ability not only to transport melanosomes within the cell, but also to export their melanosomes to the surrounding keratinocytes (Hadley and Quevedo, 1967; Zuasti et al., 1998) This enables a long term darkening of the skin around the melanophore, as the melanin is irreversibly trapped in the keratinocyte until it is broken down or the keratinocyte is shed from the skin. Melanin transfer has recently been detected in the dermis of *Xenopus* but this may be related to other biological functions than colour change as it would be obscured by the secretory glands. Melanisation seems although to have other

important functions. Melanisation is a part of the immune response in many invertebrates where pathogens are often encapsulated and the toxic intermediates in the synthesis of melanins are used as an antibiotic within the encapsulation site (Cerenius and Soderhall 2004). Melanin is also present in melanomacrophages in fish and can be seen as dark areas around wounds and sites of infection. These cells are producing their own melanin, and it might also be involved in killing invading organisms (Haugarvoll et al., 2006).

The antibiotic effect of melanin has been proposed as an additional function for human skin pigmentation. Some would even argue that this is the primary function and not UV-protection which is the most common explanation. One argument is that skin cancer is not fatal us until we are well beyond reproductive age. (Elias et al., 2009) Regardless of the reason, human melanocytes are on the epidermal side of the basal lamina separating the dermis from the epidermis. Each melanocyte contacts a large number of keratinocytes through dentritic processes enabling export of melanosomes to the keratinocytes (Tolleson 2005). Human skin colour is mainly dependent on transfer of melanosomes as human melanocytes do not perform the rapid, bidirectional transport used by fish and amphibian melanophores, even if the cells have many molecular components in common (Aspengren et al., 2009). The skin pigmentation can be increased to a darker skin colour by UV light resulting in a tanning reaction where the transfer of melanosomes to keratinocytes increase (Lin and Fisher 2007). As the keratinocytes in the epidermis are constantly moving upwards in the skin and shed, the tan will eventually be lost concomitantly with the loss of the heavily melanised keratinocytes. Transfer of melanosomes is also important for colouration of hair as they are transferred from melanocytes in the hair follicle to the keratinocytes forming the growing hair (Tolleson 2005).

Melanin is, however, still a puzzling substance. It is present in many different parts of the body, e.g. in the RPE (Retinal Pigment Epithelial) cells in the eyes, in the brain, ears (Boissy and Hornyak 2006) and has recently been discovered in the mouse heart (Brito et al., 2008). Further studies are clearly needed to elucidate the different roles of melanin, but this thesis is focussed on the role of melanin in body colouring.

The melanosome

The most striking feature of the melanophore are the dark melanosomes; each cell contains thousands of these organelles. Each melanosome is approximately 500 nm in diameter and has a core of a melanin polymer surrounded by a membrane. The melanosome is spherical to oval in shape. However, size and shape differs among species and even skin types. The membrane contains proteins from the endoplasmatic reticulum, coated vesicles, endosomes and melanosome-specific proteins necessary for melanin synthesis (Hearing, 2007). Melanosome maturation is divided into distinct stages. At stage 1, a non- pigmented vacuole is present containing intralumenal vesicles and at stage 2, melanosome fibrillar structures are evident. These fibrils are manufactured through polymerization of the Mα subunit from the Pmel17/gp100 protein and are structurally similar to the amyloid fibers formed in Alzheimer's or Parkinson's disease (Raposo and Marks, 2007). Via delivery of the major proteins for melanin synthesis, tyrosinase and tyrosinase-related protein 1 (TRP1) from early endosomes, the melanosome enters stage 3, and the production of melanin becomes evident as the fibrils are covered by the dark melanin in the mature stage 4 melanosome. Melanin is considered to be a structurally complex polymer synthesized from conversion of tyrosine to

DOPA (L-3, 4-dihydroxyphenylalanine), oxidized to DOPAquinone which reacts further in a series of events to form either the black eumelanin or the red pheumelanin. Tyrosinase is the major enzyme in the formation of melanin, but TRP1 and dopachrome tautomerase also serves important roles in the reaction. In the final stage the melanin polymer is present in the entire melanosome and the fibrillar structure of stage 2 and 3 is no longer evident (Raposo et al., 2005) As malfunction or defects in trafficking of any protein necessary for melanin production will produce a non-pigmented phenotype, mouse coat-colour mutants provide excellent tools for discovery of pigment-related genes (Bennet and Lamoreux, 2003). Many of these genes are related to human conditions such as Hermansky-Pudlak syndrome (HPS). The zebrafish genome is sequenced and the isolation of a large number of zebrafish pigment mutants give further tools in understanding the melanosome development and function. One such example is the identification of SLC24A5 in the hypo-pigmented *golden* zebrafish mutant. The gene encodes a putative cation transporter on the melanosome surface and comparisons have been made with the human gene. Interestingly, variations in the gene sequence and functionality have been found between European, Asian and African human populations that would imply that the gene have a role in the degree of human pigmentation (Lamason et al., 2005)

Cytoskeleton and molecular motors

The melanosome needs, in addition the proteins needed for melanin synthesis, a mechanism for proper positioning within the cell. In fish melanophores, melanosomes are rapidly transported from the cell periphery to the center and then back to the periphery in order to caused rapid color change. In mammalian melanocytes a rapid melanosome transport is lost and the focus seems to be on sorting melanosomes thus preventing the transfer of immature melanosomes to surrounding keratinocytes. The basic principle for the intracellular transport of melanosomes is the use of cytoskeletal components, microtubules and actin filaments, as a rail and molecular motors on the melanosomes, kinesin, dynein and myosin as a means for regulated transport.

Intact microtubules (MT) are essential for long range transport of melanosomes as both aggregation and dispersion is inhibited when MT are disrupted. MT are radially arranged from the cell center to the cell periphery and are composed of α- and β-tubulin dimers. The MT maintain a structural polarity with the growing plus end in the periphery of the cell and the minus end directed toward the cell centre. MT can undergo modifications such as tyrosination, acetylation, polyglutamylation, phosphorylation, polyglycylation and carbonylation (Hernebring et al., 2006; Ludueña, 1998; MacRae, 1997; Verhey and Gaertig, 2007) and at least polyglutamylation influence transport in melanophores (Klotz et al., 1999).

Kinesins are the motors responsible for transport of melanosomes to the MT plus-ends found at the cellular periphery (anterograde transport). Several different kinesins exist and seem to have different cellular functions. In *Xenopus* melanophores the responsible kinesin is kinesin-II (Tuma et al., 1998) and in human melanocytes conventional kinesin is involved (Vancoillie et al., 2000). These kinesins are composed of two heavy chains and two light chains. The light chains form the cargo-binding domain and the heavy chains form a coiled-coil stalk with a motor domain in the N-terminal. The motor domain consists of two MT-binding regions, one

on each heavy chain, joined by a linker region. The binding region can thus be described as feet and legs and the linker region as a waist. This allows the feet to sequentially bind to the MT, detach from the MT, rotate over the linker region and reattach in front of the other foot resulting in 8 nm steps along the MT. This motion is driven by hydrolysis of ATP and is primarily directed towards the MT-plus end. However, kinesin is able to walk backwards if faced with an opposing mechanical force exceeding 7 pN. (Gennerich and Vale 2009)

Dynein is responsible for transport towards the minus-ends of MT in the cell center (retrograde transport). It is a 1.2 MDa protein complex consisting of two heavy chains and several associated chains. The heavy chains have six ATPase domains arranged in a ring and the MT binding domain is located on a 15 nm stalk protruding between ring four and five. During ATP hydrolysis, the two heavy chains will move along the MT in steps of 4-32 nm (Gennerish and Vale, 2009). There is a reasonable explanation for the variable step size and odd arrangement with stalks instead of just having a simple "kinesin in reverse" for anterograde transport. Recent studies has shown that tau, a so called MAP (microtubule-associated protein) will inhibit kinesin transport when bound to MT, which is not found for dynein transport (Vershinin et al., 2008). This may be due to MAP tau working as an obstacle preventing transport by kinesin but allowing the passage of dynein due to the stalk-configuration and higher flexibility. The mechanism of action for how tau is able to change role is so far unclear. *Xenopus* melanophore dynein has a specific light intermediate chain enabling different regulation of dynein activity compared to other dynein-dependent cargo such as mitochondria (Reilein et al., 2003). Dynein is coupled to melanosomes via binding to the p150/Glued subunit of dynactin on the melanosome. Intriguingly, kinesin is also able to bind to dynactin but both kinesin and dynein cannot bind dynactin, simultaneously thus providing a potential mechanism for coordination of MT-transport. (Deacon et al., 2003; Gross, 2003)

Long-range transport is dependent on MT and the dynein and kinesin motors, but melanophores and melanocytes also utilize a more short range transport on actin filaments which is dependent on the myosin Va motor. In *Xenopus* melanophores this system is essential for maintaining the dispersed state or melanosomes and in melanocytes it is essential for melanosome export. Myosin Va is a homodimer and each monomer is composed of an ATP-hydrolyzing motor domain, a neck domain with regulatory sites and a variable tail domain for cargo binding variable to the myosin type (Seabra and Coudrier 2004). Myosin Va binds melanosomes via melanophilin and Rab27a. Several Rab proteins play an interesting part in melanosome regulation; Rab7 co-localizes with early melanosomes (Jordens et al., 2006) and is known to recruit dynein and dynactin to vesicles via RILP (rab interacting lysosomal protein), thus providing a means for regulation of retrograde transport (Jordens et al., 2001). Rab32 recruits PKA (protein kinase A) to *Xenopus* melanosomes thus providing a mean for spatial selectivity for PKA activity (Park et al., 2007).

The myosin Va neck contains twelve binding-sites for CaM (calmodulin) or CaM-related light chains. The binding of CaM to myosin stabilizes the neck region and prevents the molecule from folding to its inactive state. Elevated Ca^{2+} concentrations induce release of CaM and a destabilization of myosin resulting in detachment from actin filaments (Nguyen and Higuchi, 2005). Depletion of Ca^{2+} will also lead to a decreased transport and it has been suggested that

it is the binding of cargo that is the main regulator and not activation of myosin by Ca^{2+} (Sellers et al., 2008). Myosin is active in the absence of Ca^{2+} and can transport free actin filaments, while myosin is bound to a surface *in vitro*. However, myosin with a free cargo-binding region will not move on an actin filament matrix (Sellers et al., 2008). An alternative function for CaM binding and release to myosin could be binding to syntaxin 1A. Myosin Va bound to synaptic vesicles will, after CaM release, bind to syntaxin 1A on the plasma membrane thus providing a mechanism for detachment from the actin filament network to attachment to the membrane in one single step in response to an increased Ca^{2+} concentration due to ion-channel opening (Eichler et al. 2006).

Regulation of melanosome transport

As the melanosome maintains a vast array of surface proteins for transport, it is clear that they need to be coordinated in some manner to fulfil their function in the organism. To adapt to the environment, the organism need a sensor to register the appropriate external stimuli. The sensor needs to communicate with the melanophore and the melanophore needs a system to be able to respond to the signal in a robust and timely fashion for the appropriate duration. These systems show a remarkable evolution in a "use it or lose it" fashion. Teleosts have multiple and fast systems for light detection and background adaptation while amphibians adapt much slower. In humans, the ability to adapt to the background has been lost, but the ability to increase the amount of melanin and transfer to surrounding cells upon exposure to high levels of UV radiation is prevalent as discussed as discussed in the section *melanosome export*. Photoreception and response have been detected in tissues such as lateral eyes, pineal gland, iris, skin, as well as in other tissues and organs (Peirson et al., 2009).

The lateral eyes in vertebrates contain at least three types of photoreceptor cells: rods, cones and ipRGC:s (intrinsically photosensitive retinal ganglion cells). Some teleosts also possess photoreceptive horizontal cells (Cheng et al., 2009). All the cell types use different classes of opsins to absorb incoming photons and convert the reaction to a biological response. Tthere are 15 classes of opsins they are all 7-transmembrane G-protein coupled receptors and they rely on the binding of retinalaldehyde which is converted from *cis* to all-*trans* retinal when interacting with an incoming photon. This reaction triggers the G-protein transducin (Gt) which is coupled to the opsin, whereafter transducin activates cGMP phosphodiesterase. This results in a decrease in intracellular cGMP thereby inducing hyperpolarization of the plasma membrane by closure of cGMP-dependent ion channels (Bowmaker 2008). Colour vision is provided by the different sets of opsins on individual cones making them sensitive to photons with specific wavelengths. The rod cells contain the aptly named rhodopsin. They are highly sensitive and predominantly used for vision in dim light conditions. The ipRGCs are not involved in image forming vision, but are mainly responsible for pupillary response. They regulate circadian cycles in response to illumination via connections to the suprachiasmatic nuclei in the brain. The photoreceptor on ipRGCs is melanopsin (Peirson 2009). The pineal gland is also involved in circadian rhythm and produces the hormone melatonin in a photoperiodic manner. It is not photo-receptive in mammals, but very active in non-mammalian vertebrates. It is localised near the surface of the brain and as teleosts often have a thinner skull near the pineal gland, the illumination can be substantial. The photoreceptors rod and cone opsins, pinopsin, VA-opsin, exo-rhodopsin and parapinopsin have all been found in the pineal gland. The iris seems to have an another photoreceptor. It has been found that

isolated iris may retain a restriction response to light and it was suggested to be caused by melanopsin. Photoresponse by chromatophores in the skin of fish and amphibians was shown to be mediated by melanopsin in some cases (Provencio et al., 1998), but also by green and red opsins (Ban et al., 2005). Although humans seem to lack a dedicated photoreceptor in the skin, high levels of UV radiation will trigger a tanning-response. The mechanism of action for this is discussed in the section melanosome export.

Isolated tissues of different origin from *Drosophila* and zebrafish have been shown to maintain a photo-entrainable circadian clock. Photo-receptors suggested to be involved are teleost multiple opsin (Moutsaki et al., 2003) or cryptochromes (Cermakian et al., 2002).

After photoreception, the signal is transduced to the melanophores. The central regulation on photoresponse can be mediated either through nerves or by hormones. The nervous regulation is essential for fast colour adaptation and the melanophores are stimulated by the release of noradrenaline (NA) from sympathetic neurons in the vicinity of the melanophore. Nervous control of vertebrate melanophores is mainly present in teleosts and some reptiles. Poikilothermal vertebrates in general use the release of hormones from the pituitary and pineal gland to regulate melanophores (Bagnara and Hadley, 1973). Major hormones in involved are α-MSH as a signal for dispersion, and MCH and melatonin as signals for aggregation (Fuji 2000). Paracrine factors are also involved such as prostaglandins, opiates, endothelins and nitric oxide (Fuji 2003). The release of α-MSH from epidermal keratinocytes is one of the major regulators in human pigmentation (for further reading on regulation in human melanocytes see Slominski et al., 2004). As photoreception is present directly on melanophores, it will enable a localised response depending on the illumination on a specific area (Bagnara and Matsumoto 2006).

Signal transduction

The major signals involved in melanosome transport are dependent on GPCRs on the cell membrane. GPCR´s is the largest group of plasma membrane receptors and >1% of all genes in the human genome encodes these receptors (Takeda et al., 2002; Fredriksson et al., 2003). The vast number of receptors reflects the need for recognition of different ligands and they all signal, primarily, through activation of heterotrimeric G-proteins. The G-protein complex contains three subunits; termed α, β and γ. The β and γ subunits do not dissociate under normal conditions and are therefore termed $G_{\beta\gamma}$. (Johnston et al., 2007) Agonist binding to the GPCR induces a conformational change that leads to the exchange of GDP to GTP on the G_α subunit. The G_α then dissociates from the $G_{\beta\gamma}$ subunits and is free to interact with downstream effectors in the signal cascade. Inactivation of G_α is mediated by the hydrolysis of GTP to GDP and this reaction can be enhanced by a superfamily of RGS (regulators of G-protein signalling) proteins. The $G_{\alpha\beta\gamma}$ is then ready for recycling. The $G_{\beta\gamma}$ subunit could be viewed as just an inhibitor but $G_{\beta\gamma}$ have signalling properties of their own (Andersson et al., 2003). The G_α subunits can be further classified into the subfamilies $G\alpha_{i/o}$ Gαs Gαq and $G\alpha_{12/13}$ depending on their downstream effectors. (Offermanns, 2003). Melanosome transport in melanophores is mainly regulated by Gαs, $G\alpha_{i/o}$ and Gαq signals and for simplicity they will be termed here as Gs, Gi and Gq.

Gs-type receptors increase the activity of adenylate cyclase thus increasing the intracellular concentration of cAMP. This will activate PKA leading to subsequent signals that increase

kinesin and myosinVa activity while decreasing retrograde transport by dynein that finally results in dispersion of the melanosomes. Gi-type receptors inhibit the Gs coupled pathway by inhibition of adenylate cylcase thus suppressing each subsequent step. The end result is a decreased transport by kinesin and myosin while dynein transport increases resulting in aggregation of melanosomes (Rodionov et al., 2003, Gross et al 2002, Zaliapin et al., 2005). Further regulation is possible via Gq type receptors. The Gq subunit activates phospholipase C (PLC) which in turn hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP2) to inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG). IP3 opens Ca^{2+}-channels on the ER which increases the Ca^{2+} concentration in the cytoplasm whereafter DAG and Ca^{2+} activate PKC. In *Xenopus* melanophores, this reaction results in dispersion of the pigment (Sugden and Rowe, 1992). The increased Ca^{2+} concentration has most probably more functions such as modulation of calmodulin binding to myosin-Va. In a recent study (Isoldi et al., 2009), light was found to induce signalling via the Gq pathway. This increased calcineurin (PP2B) activity which inhibited α-MSH-induced cAMP increase in *Xenopus* melanophores. The inhibition is caused by inhibition of adenylate cyclase by PP2B and/or PKC providing a mechanism for crosstalk between signalling pathways (Isoldi et al., 2009). Adenylate cyclase modulation, is in turn, determined by which one of the nine isotypes of adenylate cyclase expressed by the cell (Isoldi et al., 2009).

$G\alpha_q$, $G\alpha_i$, $G\alpha_s$ and $G_{\beta\gamma}$ subunits may also directly influence MT-stability by binding to binding to tubulin (Dave et al., 2009). Furthermore recent developments in GPCR-structural research suggest that many receptors do not have only one specific signalling cascade, but have higher or lower efficiency in the different pathways depending on modifications, binding to ligands, oligomerization and membrane compartmentalization (Rosenbaum et al., 2009).

Melanosome export

Amphibians use melanosome export and transfer of pigment to keratinocytes as a long-term adaptation to the surrounding environment, but this can also be combined with a rapid intracellular dispersion of melanosomes (Hadley and Quevedo, 1967). Fish have another strategy; species living with a dark or light background will increase or decrease the number of melanophores to obtain long-term adaptation (Sugimoto, 2002). Fish raised in shallow waters can respond by increasing the amount of melanophores upon UV radiation (Adachi et al., 2005). Although studies exist on melanosome transfer in amphibians (Aspengren et al., 2006a; Hadley and Quevedo, 1967) most of the research on melanosome transfer is performed in mammalian models

As mentioned previously, skin and hair colour in mammals is dependent on the export of melanosomes. High transfer of melanosomes results in a dark colour and a low deposition will result in a lighter or white colour (Sarin and Artandi, 2007). The term constitutive pigmentation is used for the basal level of skin pigmentation without exogenous stimulants like UV, whereas the term facultative pigmentation is used for pigmentation after exogenous stimulation (Rouzaud et al., 2005).

The main function for pigment transfer in humans seems to be the protection against the damaging effects of UV radiation (Miyamura et al., 2007). The radiation causes DNA damage

thereby increasing DNA-repair mechanisms, but it also affects NAD, quinones and flavins, causing peroxidation of lipids in cellular membranes which leads to production of reactive oxygen species (ROS) (Kochevar, 1995; Sies and Stahl, 2004). Peroxidized lipids have previously been shown to induce release of diacylglycerol (DAG), resulting in activation of PKC (Nishizuka, 1986). DAG is involved in increasing melanogenesis, thereby acting synergistically with UV radiation (Gordon and Gilchrest, 1989). UV radiation does not only affect melanocytes, but has several effects on keratinocytes as well. It leads to the release of several growth factors and cytokines such as α-MSH, ACTH, NO, and ET-1 (Costin and Hearing, 2007; Rouzaud et al., 2005; Chakraborty et al., 1995; Rouzaud et al., 2005). α-MSH binds to the MC1R receptor on the melanocytes, resulting in an increase in cAMP, PKA activity, and expression of the microphtalmia-associated transcription factor, MITF (D'Orazio et al., 2006). The expression of MITF has several effects on the melanocytes, it leads to an increase of expression of proteins involved in melanin synthesis but also to cell proliferation. The MCR1 receptor plays an important role in skin and hair colour. Several mutations are known that reduces ligand binding to the MCR1-receptor, leading to pheomelanin production instead of eumelanin production. The resulting phenotype is red hair, fair skin and an increased risk for skin cancer (Han et al., 2006).

Several models have been put forward of which exocytosis of the melanosome followed by phagocytosis by the keratinocyte is one. Keratinocytes have been suggested to use cytophagocytosis to engulf melanosome-containing dendrites from the melanocyte or to fuse plasma membranes between the cells to form a channel for melanosome transfer. There is also evidence for transfer via membrane-enclosed exosomes containing one or more melanosomes (Aspengren et al., 2006a; Boissy, 2003; Jimbow and Sugiyama, 1998; Marks and Seabra, 2001; Seiberg, 2001; Van Den Bossche et al., 2006). Even if they have been presented as different models, it might be that several of these mechanisms exist in the same organism or that mechanisms differ among species, tissues or even age. Different methodological approaches have furthermore been used by different researchers which might affect the conclusions. The molecular players suggested so far will be discussed below.

Regulators of dendricity and transport

Melanocytes are highly dendritic, and because of the dendricity, they can be in contact with several keratinocytes. Melanocyte dendrites are dynamic and the formation is dependent on actin polymerization. This means that actin filaments are not only involved as tracks for melanosome transport within melanocytes, but also for the ability of the melanocyte to transfer melanosomes to as many keratinocytes as possible. Actin polymerization is highly regulated by members of the GTP-binding proteins Rac, Rho and Cdc42 which induce lamellipodia, stress fibers, and filipodia respectively (Etienne-Manneville and Hall, 2002). UV radiation plays a role and stimulation of melanocytes with either UV radiation or α-MSH activates Rac, decrease Rho activity, and increase dendrite formation possibly via cAMP signalling (Scott et al., 2002, 2003). In addition, UV stimulated release of prostaglandin from keratinocytes induces dentricity in melanocytes by binding to the EP1 and EP3 receptors and PKCζ activation (Scott et al., 2007).

To reach the dendrites and the cell membrane, the melanosomes are transported along the actin filaments by the myosin-Va motor that is bound to the melanosome via Rab27a and

melanophilin. Mutations in any of these proteins affects the ability to form a proper complex and will result in the Griscelli syndrome I-III in humans or the *dilute*, *ashen* or *leaden* in mouse (Van Gele et al., 2009). Melanosomes lose the ability to be transported to the dendritic tips and remains in the perinuclear region of the melanocyte resulting in an inability to transfer melanosomes. Humans with this mutation have silvery grey hair colour and a discrete hypopigmentation, and mouse coat colour of is lighter. The coat color of the *dilute* mouse can be become partially restored in the loss of function *dilute suppressor* (*dsu*) mutant. Even though the melanosomes are retained in the perinuclear area of the melanocyte, melanosomes can be transferred into the hair shaft, but they will be not be evenly distributed suggesting a myosin-Va independent transfer (O'Sullivan et al., 2004). The dilute suppressor protein, melanoregulin, has recently been suggested to function as an inhibitor of membrane fusion by blocking peripherin-2 (Boesze-Battaglia et al., 2007), which might explain the clumping of melanosomes. It might be that the melanosomes are transferred via the membrane of the cell body instead of the dendrites, a mechanism which is repressed in the wild type but not in the *dsu* mutant lacking the functional protein. With no melanosomes in the dendrites of the melanocytes, fewer keratinocytes can be accessed for transfer so only keratinocytes in the very proximity of the melanocyte will become pigmented which way explain the clumping of melanin in the growing hair.

Advanced methodology has been used to address the role of myosin-Va. A perinuclear aggregation of melanosomes was induced when RNAi specific for myosin-Va F exon was used in human melanocytes (Van Gele et al., 2008). The results open up for a future possibility to use viral delivery of the interfering RNA to treat hyperpigmentary disorders. Perinuclear aggregation of melanosomes is also seen upon silencing of MITF. The effect is partly mediated by the downregulation of Rab27a expression. Restoration of MITF expression restores both the expression of Rab27a expression and the ability of melanosomes to transport to the cell periphery (Chiaverini et al., 2008). These effects are not limited to mammals as downregulation of MITF in *Xenopus* decreases Rab27a expression and induces aggregation of melanosomes, an effect that is reversible upon restored expression of MITF (Kawasaki et al., 2008). MITF seems to be the central actor in melanocytes; it is involved in melanocyte differentiation, cell-cycle progression and survival (Yamaguchi et al., 2009), and now a role in the expression of transport-related proteins such as Rab27a can be added.

Exocytosis

The presence of SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptors) proteins and Rab GTPases in melanocytes indicates that they play a role in the exocytosis of melanosomes at cell membrane. The proteins are well known to play a part in membrane fusions between a cellular organelle and the cell membrane; In nerve cells, neurotransmittors are transported on organelles that with the use of SNAREs, dock into the membrane, and empty the neurotransmittors, whereafter the organelle membrane is reused within the nerve cell (Jahn and Südhof, 1999). The evidence for a similar mechanism in melanocytes/melanophores is, however, not strong, even if there are some observations that might support it. Membrane-free, extracellular melanin granules have been found by electron microscopy in human hair and skin (Swift, 1964; Yamamoto and Bhawan, 1994). This could however, be an artefact from the preparation for EM, as EM experiments on melanophores indicate that the membranes may be lost in the fixation procedure (Aspengren and Wallin

2004). The membrane marker DiI is co-localized with exported melanosomes (Aspengren et al., 2006).The evidence for transfer of membrane-enclosed melanosomes is, however, stronger. Studies show transfer of membrane-bound tyrosinase (Cardinali et al., 2005; Lin et al., 2008). Further studies are needed to evaluate if melanin can be exocytosed, both with and without its membrane.

The expression of of SNARE proteins such as Rab3a, VAMP-2, SNAP23, SNAP25, and syntaxin 4 suggests that they are involved in some way even if the mechanism is not known, and even if it might differ from what is known from e.g. neurons (Araki et al., 2000; Scott and Zhao, 2001). They are not only present, but when melanocytes are treated with α-MSH which increases transfer of melanosomes, the expression of several SNARE-proteins is increased (Virador et al., 2002). Another role might be to be responsible for the fusion of membranes in melanosome biogenesis rather than the process of exocytosis.

Cell recognition and phagocytosis

The G-protein-coupled transmembrane receptor PAR-2 (protease-activated receptor-2) is present on keratinocytes and in many tissues (D'Andrea et al., 1998; Macfarlane et al., 2001; Derian et al., 1997; Santulli et al., 1995), but absent in melanocytes (Seiberg et al., 2000; Sharlow et al., 2000). It is activated by a conformational change induced by cleavage of the extracellular domain by serine proteases. Activation of PAR-2 has many effects on human keratinocytes. It induces Ca^{2+} mobilization (Böhm et al., 1996), rearranges the cytoskeleton and induces morphological changes of the cell surface, and increases phagocytotic activity (Sharlow et al., 2000). This activation is clearly connected to phagocytosis, it induces an increased uptake of melanosomes or fluorescent latex beads (Macfarlane et al., 2001; Scott et al., 2003) The phagocytotic activity can be decreased by inhibition of the intracellular signalling mediators Rho and Rho kinases (Scott et al., 2003).The PAR-2 receptor is affected by UV radiation, both its activity and expression (Scott et al., 2003) and it has most probably a role in the different colouring of human skin as the activity and expression is higher in dark skin compared to light skin (Scott et al., 2001). The effects of activation of PAR-2 is not only restricted to keratinocytes, it triggers the release of prostaglandins (PG) which stimulates the dendricity of the melanocytes via a cAMP-independent pathway (Scott et al., 2003) thereby increasing the efficiency of melanosome transfer from melanocytes. Phagocytosis can further be increased by secreted factors from skin cells. The keratinocyte growth factor/fibroblast growth factor 7 (KGF) is one such an example which is mediated both by the Rho and the Cdc42/Rac pathway (Cardinali et al., 2005).

Melanocyte membranes trigger a transient release of intracellular Ca^{2+} stores in keratinocytes, and chelation of Ca^{2+} decreases pigment transfer (Joshi et al., 2007), indicating the presence a membrane recognition mechanism. Lectins are receptors that recognize sugar residues on glycoproteins bound to the cell membrane (Monsigny et al., 1988). After UV exposure, keratinocytes increases the expression of surface lectins specific for alpha-L-rhamnosyl or alpha-D-glucosyl residues (Condaminet et al., 1997). Addition of lectins or neoglycoproteins to melanocyte-keratinocyte cell cultures results in a decrease in transfer of melanin in a reversible manner (Cerdan et al., 1992; Greatens et al., 2005; Minwalla et al., 2001a). Lectins on melanocytes are mainly specific for α-L-fucose, and small extracellular melanin-

containing vesicles have receptors specific for 6-phospho-β-D-galactosides (Cerdan et al., 1992), whereas keratinocytes specifically bind glycoproteins with α-L-fucosyl or α-L-rhamnosyl residues (Cerdan et al., 1991). We have been able to reduce melanin transfer in our *Xenopus* assay by adding mannose-binding lectins from *Pisum sativum,* but not with galatose- or α-L-fucose binding lectins (unpublished data), suggesting that a role for lectins may have evolved early.

Melanosome positioning within keratinocytes

Melanosomes are present in keratinocytes either as single melanosomes or as groups of melanosomes covered with a membrane (Yamamoto and Bhawan, 1994, Aspengren et al., 2006). Dark-skinned individuals have relatively large melanosomes present as single organelles, while fair-skinned have smaller melanosomes clustered in phagosomes (Minwalla et al., 2001b; Thong et al., 2003). Experiments with uptake of latex beads in keratinocytes show that large beads are stored as clusters and small as individuals suggesting a size-dependency (Virador et al., 2002). However, many factors are probably involved. It turns out that when melanocytes and keratinocytes are mixed from different skin types, the origin of the keratinocytes determines whether melanosomes are clustered or not (Minwalla et al., 2001b; Yoshida et al., 2007). Clustered melanosomes can also be disassembled to individual melanosomes to form a nuclear cap as shown by the use of EM (Okazaki et al., 1976). Many details remain to uncover regarding uptake, sorting and transport in keratinocytes but it is clear that dynein- and dynactin $p150^{glued}$ are important players to achieve a supranuclear cap of melanosomes in human melanocytes since, since knock-down of dynein heavy chain or $p150^{glued}$ disperse melanosomes evenly (Byers et al., 2007; Byers et al., 2003).

AIMS OF THE THESIS

The aims of this thesis were to increase the understanding of pigment transport and transfer, and its regulation in the amphibian *Xenopus laevis,* and in zebrafish, *Danio rerio*, as well as to further explore whether melanophores can be used for toxicological studies. To achieve these goals it was necessary to improve old methods and and develop new.

More specific aims were to:

- Examine how transport and transfer of melanosomes are performed in a co-culture of *Xenopus laevis* melanophores and fibroblasts.
- Develop a new fast method to quantify transfer of melanosomes.
- Explore the possibility of using cultured *Xenopus laevis* melanophores for toxicological studies.
- To develop cell culturing techniques of melanophores from zebrafish in order to further explore their regulation and function.

METHODS

Animals

Zebrafish were purchased from local suppliers and kept in aquaria at the department of Zoology at the University of Gothenburg. The aquaria were housed in a compartment for tropical fish at 27 °C and a 12/12 day/night cycle with 60 minutes of simulated dusk/dawn. Siphoning and removal of debris was done weekly and feeding commercial fish food 1-2 times a day depending on energy demands due to breeding and egg production. Egg collection was done by placing breeding chambers in the aquaria the day before collection. The chambers float in the water and allows for the fish to enter from the side. The floor of the chamber contains openings large enough for eggs to pass to a closed compartment inaccessible for the fish. This is important since the eggs are often eaten by the adults. Mating behaviour is initiated at dawn so collection of fairly synchronized eggs was possible 30-60 min after the light was turned on. Skin preparations in **Paper I** were made from the dorsal skin of adult *Xenopus laevis* kept at the department of Zoology at the University of Gothenburg. The frogs were anesthetised in MS222 and decapitated before sampling.

Xenopus melanophores and fibroblasts

The melanophore cell line used was established in 1990 (Daniolos et al., 1990) and provided to us by Dr M. Lerner, Arena Pharmaceuticals Inc. (San Diego, CA, USA) together with *Xenopus* fibroblasts which are necessary for conditioning of the melanophore media. The cell line was established by triturating *Xenopus* tadpoles at stage 30-35 (Nieuwkoop and Faber, 1967) and then culturing the cells at 27 °C in medium conditioned by *Xenopus* fibroblasts for 3-4 days. Under these conditions colonies of melanophores were found to grow in the primary cultures and Daniolos et al, 1990 isolated the melanophores by Percoll centrifugation during subcultivation of the cells. Since these original cells were isolated from a population of cells from several individuals rather than from a single clone, the culture is quite heterogenic. The characterisics of the cells in the culture is also affected by the culture conditions. If the cells for example are repeatedly cultivated at low densities the culture will be dominated by a less pigmented, but faster growing sub population(Suska et al., 2008). This can be minimized by subcultivating at high densities and occasional Percoll gradient centrifugations. While this cell line is highly sensitive to storage at -80 °C they can be put in a semi active state for long term storage at 17 °C in regular culture flasks.

Murine melanocytes and keratinocytes

The mouse melanocyte line melan-a (Bennett,et al., 1987) was supplied from the Wellcome Trust Functional Genomics Cell Bank (St George's, University of London, UK). The cell were

established from C57BL/6J mouse and are cultured in RPMI 1640 supplemented with 100 units/ml penicillin, 100 mg/ml streptomycin, 2 mM glutamine, 7.5 mg/ml phenol red, 10% FCS and 200 nM TPA (12-*0*-tetradecanoyl phorbol acetate) at 37 °C and 10% CO2. The cells were subcultured after 7-10 days and the medium was exchanged once a week. Mouse keratinocytes XB-2 (Rheinwald JG, Green H. *Cell* **6**, 317-330 (1975)) were supplied from the Wellcome Trust Functional Genomics Cell Bank (St George's, University of London, UK). The cell line was established from a mouse teratoma. They were cultured in DMEM supplemented with penicillin (100 units/ml), streptomycin (100 mg/ml) and L-glutamine (4 mM) and 10% FCS at 37 °C and 10% CO2. The cells were subcultured after 7-10 days and the medium was exchanged once a week.

Zebrafish cultures

The advantage of having a melanophore cell line is the constant availablity of cultured cells, and that enough cells of the same origin can be cultured for biochemical and molecular biological studies. Primary cultures of cod melanophores have previously been used in the laboratory, but a decision was made to change to Zebrafish due to the advantage of its sequenced genome. The work started with establishing a melanophore primary culture from zebrafish embryos, a culture that is a mix of different cell types. The melanophores were found to be dependent on the non-melanophore cells since they did not attach or proliferate well in their absence. In order to facilitate the process of establishing a melanophore cell line we have continuously cultivated what appears to be an immortal population of cells from the primary cultures since 2004. We have used them as feeder cells (after mitomycin treatment) for melanophores and for conditioning of the culture media similar to the methodology for culturing of Xenopus melanophores. While they did enhance attachment of melanophores, possibly by secretion of proteins on the surface of the culture dish, no significant cell division of the melanophores was observed. The embryonic cells were cultured in zebrafish media; L-15 supplemented with 10% FCS, penicillin (100 units/ml), streptomycin (100 mg/ml), 2 mM L-glutamine, 1x antibiotic-antimycotic and 0.5 mM mM $CaCl_2$. They were grown at a constant temperature of 27 °C, but could also easily be grown at room temperature. They are now sub cultured weekly and the media is exchanged once a week. They are extremely tough and can survive several weeks without passages or change of culture media. We have 7 different primary culture variants in passage 2-8 frozen in -80 °C. The present cells were established 2004-05-17, frozen 2005-07-14 at passage 8, thawed 2006-10-31 and have been cultured since in 2 separate cultures termed I and II. In the early stages the cells were sub cultured when they reached confluence and not weekly as we do now. They test positive against a pan-reactive cytokeratin (Panreactive C-11, Biolegend) antibody both in WB and IC. We therefore term them Zebrafish embryonic epithelial (ZEE) I and II until further classification is done.

Cell cultures from fins or split fin preparations have been a traditional approach for studies of chromatophores in fish. Fin preparations were used in **Paper IV** to analyse melanophore behaviour in response to light as described in detail in **Paper IV**. In brief, adult zebrafish was sacrificed by decapitation and fins were removed, washed and dissociated with forceps. The pieces were allowed to attach to the surface of culture plates in drops of zebra fish media for 2-4 hours before adding the final volume of zebra fish medium. After a few days unpigmented cells migrated from the attached fin and formed a monolayer of cells surrounding the fin. The melanophores then migrate on top of the monolayer but not beyond the edges. The main

problem we experienced in **Paper IV** was the release of melanosomes to the unpigmented cells thus making quantification of aggregation/dispersion in melanophores hard to quantify with the image analysis software since it depends on having a bright and contrast free background to the melanophores.

Scales from zebrafish were also used in **Paper IV**. They were taken from the sacrificed fish used for fin preparations,placed in zebra fish medium and photographed. The advantage of using scales is that it allows for a quick isolation and many melanophores are present. However, careful examination showed that they vary both in their response to light and drugs. The reason for this is of course interesting, but from our point of view for these studies it was disadvantageous in several ways. The degree of pigmentation in the scales changes in the dorsal-ventral axis. This can be compensated by careful sampling of scales in the same region of all fish. Within each scale there seemed to be different subpopulations of melanophores. Melanophores near the edge of the scale often behaved differently from the ones in the central region. This may be an effect of different thickness of the tissue on the scales and also caused by the overlap of scales. Cells in the central region may under normal circumstances be covered by the overlapping scale thus providing a different microenvironment compared to the peripheral melanophores. Since we wanted to measure the light responsiveness in melanophores, the shading of an overlapping scale may influence the level of adaptation and expression of receptors in a "shaded" region. Furthermore, we can not exclude the possibility that cells surrounding the melanophores could influence the melanophore in a paracrine fashion similar to the interaction between human keratinocytes and melanocytes during UV exposure. Nerve cells are known to reach and signal to scale melanophores, and even if the scales are removed from the fish active nerve endings might influence the cells.

Embryo cultures from zebra fish were extensively used in preparation for **Paper IV**. Initially we established the protocol in order to obtain a stable melanophore cell line. While primary cultures are easily obtained by the method in **Paper IV** and the melanophores can be maintained in culture for approximately 4 weeks they do not increase in number and suffer substantial losses during subcultivation. We have used several methods to stimulate division such as addition of bFGF, increased FCS, cholera toxin, phorbol ester (PMA), α-MSH, conditioning the media on ZEE I-II or on *Xenopus* fibroblasts, using ZEE cells as feederlayer or increasing adhesion by using (cell+) culturing vessels or coating of the vessel with fibronectin to improve adhesion. While in search for the X-faktor that would induce melanophore division, information of circadian rhythm became interesting since each cell in zebra fish can maintain a rhythm in response to light cycles and cell division in embryos peaks at dusk (Dekens et al., 2003). While culturing the cells at 12/12 hour light/dark cycles we could not obtain cell division in melanophores but it was clear that the cells reacted to light by dispersion and dark by aggregation of melanosomes. Similar like the fin cultures, melanophores often grow on top of the monolayer of cells surrounding pieces of tissue that adhere to the substrate. While fibronectin and the use of Cell+ culturing vessels increase the number of melanocytes that adhere directly on the substrate they did not proliferate well.
The embryos we use are either 48 hrs or 5 days post fertilization and are thus fairly small (2-4 mm). Removal of the chorion, if present, decapitation and dissociation is done with syringe needles in Ca^{2+} -free Ringers solution and the tissue is treated with 0,17% trypsin before washing and culturing in zebra fish media.

Co culturing and manual transfer quantification

In **Paper I** we developed a co-culturing protocol for *Xenopus* melanophores with fibroblasts and a method for quantification of the subsequent transfer. 20.000 melanophores were plated with 60.000 fibroblasts in 60 mm culture dishes with a grid pattern. The same cell suspension was used for all the dishes in one experiment to minimize variation in the number of cells on each plate. To enhance equal distribution of the cells, the culture plates were placed on a tray which was rocked three times back/forth side/side and then immediately and carefully placed in the incubator. This step turned out to be essential since cells have a tendency to aggregate in the centre of the dish and this tendency is enhanced by rotating motions. After 2 days the media was changed and drugs were added. After 2 days of treatment the media was removed, the cells were fixed in ice-cold methanol and washed with PBS and water. The plates were allowed to dry and could then be stored indefinitely. Determination of the area (10x10 squares in the grid) to be quantified was made by marking the spot where the methanol was added, since it would contain damaged cells, and then the opposite area was selected for quantification. The plates were then marked in such a way that the treatment was unknown to the operator performing quantification. By using bright field microscopy at low magnification it was possible to detect transferred melanin, but not the transparent fibroblasts. By increasing magnification and switching between bright field and phase contrast (to detect fibroblasts) it was possible to determine the number of melanin positive fibroblasts.

Flow cytometry and markers

The principle for flow cytometry is based on the use of a laser to analyse optical properties of a flow of single cells passing the detector. The FACSCalibur flow cytometer was used in **Paper II**. Forward scattering (FSC) estimates cell size and side scattering (SSC) estimates the presence of scattering organelles within the cell. Fluorophores could be detected at different wavelengths; FL 1 channel 515-545 nm, FL 2 channel 564-606 nm, and FL 3 channel >650 nm). Fluorophore-conjugated antibodies against cell type specific proteins was used to differentiate subpopulations within a cell suspension. Flow cytometry was performed at the Institute of Biomedicine at Sahlgrenska Academy.

Aggregation and dispersion assays

For aggregation (**Paper III**) and dispersion (**Paper I**) assays *Xenopus* melanophores were cultured in 96-well culture plates and the aggregation of the melanophores was recorded with a SPECTRAmax 190 microplater reader with Softmax PRO v.3.1.2. The cells were usually seeded at least 3 days before measurements at a density of 20-40.000 melanophores/well. Low densities allow for observations of morphology changes after treatment. High densities one the other hand generate larger changes in absorbance between aggregation and dispersion making readings easier but makes observation of morphology changes harder. In **Paper I** the cells were pre-aggregated with 10nM melatonin in serum free frog medium for 1 hour before addition of α-MSH. The absorbance at 650 nm was recorded for each well immediately after α-MSH addition and then every 10 minutes for 90 minutes. Calculation of dispersion was made as $1\text{-}10^{Ai\text{-}Af}$ (Potenza and Lerner 1992) were Ai is absorbance directly after addition of α-MSH and Af is the absorbance at the time point of interest. For the aggregation assay in **Paper III** the melanophores were pretreated with test substance in serum free frog media for 24 hrs and were thereafter allowed to disperse in room light for 2 hours prior to the assay. To

induce aggregation 10 nM melatonin was added to each well and Ai was immediately recorded at 650 nm and further readings were performed every 5 minutes for 60 minutes and the aggregation was calculated as Af/Ai-1(Potenza et al., 1994).

Image analysis by Image J

ImageJ is a Java based image analysis platform developed by NIH. It is an open source and freely available (http://rsbweb.nih.gov/ij/index.html) making it a program that is further developed by the users. In **Paper IV** ImageJ was used to analyse aggregation and dispersion in primary cultures of zebrafish melanophores. The melanophores were illuminated by a set intensity under the microscope for 30 minutes allowing the melanosomes to disperse. The dispersion state was photographed and the image was used as the initial value I. The illumination from the microscope was then blocked with a shutter and all light sources in the room were turned off for 60 minutes allowing the melanophores to aggregate. The illumination was then restored and the melanophores were photographed for every 2.5 minute for 25 minutes. The resulting image series was then exported to ImageJ and converted to a single stack of images. By using the "Enhance contrast" and "Subtract background" the melanophores could be filtered from background. By using "Adjust/Threshold" the image was converted to a binary image. Regions with melanophores were selected and "Analyze particles" was used to quantify the area covered with melanosomes in each frame of the stack. The dispersion could then be calculated as percent of the initial value for each time point. Melanophores often overlap each other so dispersion was not calculated on individual cells but on all cells in the selected region.

RESULTS AND DISCUSSION

Paper I: In vivo and in vitro transfer of melanosomes

Paper 1 established a co-culturing protocol and quantification method of pigment transfer between melanophores and fibroblasts from African clawed frog, *Xenopus laevis*. It is known that the melanophores need growth factors secreted from the fibroblasts to proliferate well. For regular culturing the growth medium was conditioned for 3-4 days on the fibroblast culture and then removed and sterile filtered before used for culturing of the melanophores. The use of practically the same media for both cell types was convenient for co-culturing of melanophores and fibroblasts, the fibroblasts supplied growth factors under more or less similar conditions as when mono cultured. The only difference would be if potential signals from the melanophores were secreted and affecting the fibroblasts, as signalling crosstalk is significant between skin cells. However, this would only represent a more normal situation compared with the *in vi*vo situation since dermal melanophores and fibroblasts do come in contact with each other in *Xenopus* dermis. When co-culturing mammalian melanocytes with keratinocytes media formulations is a problem since the mono-cultures usually grow in different media such as RPMI and DMEM. Several melanocyte cell lines, such as melan-a, also need growth factors like PMA or cholera toxin for proliferation and the presence of such factors may, in turn, have unwanted effects on keratinocytes in terms on proliferation or differentiation. It should also be considered that in a mammalian system, mixing melanocytes and fibroblasts would not represent a normal situation as the two cell types are separated by the basal lamina. They do communicate via secreted factors, and influence each other, but are not in direct contact other than during clinical conditions.

When Xenopus melanophores and fibroblast were co-cultured, the following observations were made:

1. In early cultures, melanophores display motility responses towards neighbouring fibroblasts. One could speculate that this might be due to release of chemotactic factors from the fibroblasts, cell-cell interactions or favourable surface properties of the substrate caused by protein release from the fibroblasts.
2. When cultures were almost confluent, the melanophores are often highly dendritic and the cell bodies often seem to be compressed. This could be a result of competition with the faster growing fibroblasts for attachment to the substrate.
3. It is possible to manually count and quantify transferred pigment by light microscopy without the use of any extra melanosome markers or the use of electron microscopy which differs from studies on melanocyte transfer experiments. This is possible due to the large difference in cell size and morphology between the melanophores and the fibroblasts, as well as that the vast majority of melanosomes are mature and

completely filled with eumelanin. They are therefore easy to detect and healthy fibroblasts generally lack other clearly visible organelles in the cytoplasm that might interfere with the counting of transferred melanosomes.

4. The transfer is increased by addition of 120 nM α-MSH
5. Phagocytosis of fluorescent latex beads in the fibroblasts is not increased by α-MSH in a statically significant manner.
6. Transferred melanosomes co-localize with cell trackers used to mark membranes (DiI) or the cytoplasm/proteins (CMFDA) of melanophores prior to co-culturing. This indicates that the melanin present in the fibroblasts were transferred from the melanophores to the fibroblasts and not produced within the fibroblasts.
7. By electron microscopy free membrane-enclosed melanosomes are detected both *in vivo* and *in vitro*. Free and transferred melanosomes are also found surrounded by second membrane, enclosing the melanosomes.

The transfer of the membrane dye DiI with the melanosomes and the presence of membranes on melanosomes in the EM preparations suggest that a classic, synapse-like exocytosis is not the mechanism of transfer. In synaptic exocytosis the vesicle membrane is fused either permanently or temporarily (kiss-and-run) with the pre-synaptic membrane while releasing its inner cargo. If this was the case in melanophores, the melanosome would be transferred without its membrane and this was not the case. There is also other evidence pointing in the same direction, as several studies (Cardinali et al., 2005, Lin et al. 2008) are successfully using melanosome-bound proteins as markers for transfer.

In the cell culture, the fibroblasts with transferred melanosomes were often found close together in groups. This suggests either the presence of more active phagocytotic subpopulations or that delivery of melanosomes is restricted to specific areas by the presence of melanophores. Melanophores in these co-cultures are able to form very long dentrites enabling access to distant fibroblasts. It is however not obvious from viewing the co-cultures that melanophore dendrites are present in contact with all the fibroblasts that had taken up melanosomes. However, one can not exclude the possibility that the dentrites are dynamic (as for neuronal dendrites) and retracts after delivery of melanosomes. A clear cell-to-cell contact between melanophores and fibroblasts would then not be observed for all cells, only those contacts that exist at the moment of fixation. Another mechanism for generation of the spatially clustered fibroblast could be phagocytosis of apoptotic melanophores including the cells melanosomes. This would however only contribute to a baseline transfer and not explain the increase in transfer since α-MSH is known to prevent apoptosis (Abdel-Malek et al 2008). Thus, based on paper I, there is some support for a mechanism with cell-cell interactions as it would explain the presence of groups of fibroblast with melanosomes and that phagocytosis of membrane-enclosed melanosomes is the uptake mechanism.

EM provides the resolution necessary to explore the mechanisms in detail, but suffers some drawbacks; the sample preparation is quite severe involving among else complete dehydration of the samples in pure ethanol. This will inevitably create artefacts or loss of information and may explain why free melanosomes in some studies lack membranes. Another drawback is of course that it is impossible to study single cell processes over time. Therefore it is hard to tell if the melanosomes in the fibroblasts were transferred as a group of melanosomes with one additional membrane surrounding the group that originates from the melanophore or if the melanosomes were transferred one by one and then sorted to one organelle in the fibroblast.

EM is furthermore only of two-dimensional nature; each section is approximately 60-70 nm thin and objects along the Z-axis will look the same regardless of their length. This can make dendrites look like vesicles and vice versa although this problem could be remedied by creating Z-stacks by coordinating several sections of the same object.

The results show clearly that transfer was stimulated with α-MSH. The present method can not specify any mechanism explaining the increase, but there are several possibilities. An increase could depend on any of the following alone or in combinations:

1. Increased number of melanophores due to α-MSH stimulation.
2. Increased melanogenesis within each melanophore.
3. Increased transfer due to dispersion of melanosomes to the cell periphery thus increasing their availability for transfer.
4. Upregulation of the export mechanism at the membrane.
5. Increased phagocytosis by recipient cells.

While 1-5 may be upregulated during α-MSH stimulation it would be preferable to selectively block 4 in order to explore the mechanism(s) involved. And this was the starting point for **Paper II**.

Paper II: Quantum dots as a new promising tool for studies of melanosome transfer

The notion of exocytosis or an exocytosis-like mechanism for transfer opened up a route for further experiments; the use inhibitory neurotoxins. SNARE proteins are known targets for both botulinum and tetanus toxins and SNARE proteins have been detected in melanosomal fractions (Scott et al., 2001). Treatment of co-cultures with the complete toxins was not an option as both toxins rely on specific receptors for uptake and they could potentially interfere with the fibroblasts. The solution was to transfect the melanophores prior to co- culturing with a plasmid containing only the light chain of tetanus toxin fused to EGFP (enhanced green fluorescent protein) for detection. The result would be fluorescent transfected cells and the toxic light chain would only be active inside the cell thus minimising the effect on the fibroblasts. It became, however, clear that these transfections were not optimal for such studies. Cell death due to the transfection would increase the unspecific release of melanosomes, the efficancy of transfection varied and the melanophores were extremely resistent to Geneticin making selection for stable transfectants difficult. Another drawback is that when the manual quantification method was used in **Paper I**; counting 12 plates took almost a week of microscopy, constantly shifting objectives between phase contrast and bright field imaging. Another approach and another quantification method were thus needed.

Flow cytometry can be used for making transfer analysis more efficient. This has previously been done (Minwalla et al., 2001) by incubating melanocytes with the celltracker CFDA and by using a cytokeratine antibody to detect keratinocytes. Flow cytometry appears to be a good quantification method, but CFDA will dye the whole melanocyte, no conclusions can be drawn about the origin of the membrane of the transferred melanosome. Other methods have also been tested extensively (Berens et al., 2005). The use of GFP-tagged melanosome proteins will either lead to missorting of the protein, or a low fluorescence possibly due to the low pH in the melanosome or quenching of the signal by the melanin. The use of membrane

dyes such as DiI or DiO (Aspengren et al., 2006, Scott et al., 2002) is also possible, but they have the drawback that they stain all membranes and are sensitive for fixation protocols that disrupt the membranes.

Transferred melanosomes have been detected with antibodies against the key melanosome enzyme tyrosinase concomitant with identification of keratinocytes with anti-keratin antibodies in manual transfer quantification (Cardinali et al., 2005), but this method is not very efficient. The approach of combining the use of antibodies, for cell and organelle determination, with flow cytometry appeared, however, attractive. While testing markers, it soon became apparent that only a few appropriate antibodies against *Xenopus* melanosome proteins are available. There are far more antibodies available for mouse and human proteins, so we decided to work with mouse melanocytes to test if the combined methodology was working. Mammalian melanosomes are not as dark as the amphibian melanosomes, which is another advantage. Highly melanized melanosomes quench fluorescence and mammalian melanosomes would therefore potentially interfere less with the signal.

In theory, immunocytological staining is very specific, but in reality, there are several prerequisites that must be met or considered during evaluation of the results:

1. The target protein must co-localize with the melanosome, not only in the melanocytes, but also in the keratinocytes, and it must be preserved during the experiment.
2. The antibody must be able to reach the antigen. In this case it must cross the cell membrane and the membrane of the organelle that contains the melanosome in the recipient cell. This can be achieved by permeabilizing the membrane with detergents, such as Saponin, Trition-X, but must not be so severe that the antigen changes conformation and or is destroyed.
3. The resulting signal must be significantly stronger than background fluorescence, fixation artefacts, or unspecific binding of the primary or secondary antibody.
4. The recipent cell must be identified by some marker that distinguishes it from melanocytes while not interfering with the melanosome marker.
5. Depending on the type of the flow cytometer used there may be constraints in what fluorophores are available. In a mono-laser system, such as the FACS Calibur used in this study, the fluophore must be excitable by the 488 nm Argon laser but have an emission in three different ranges, either 515-545 nm, 564-606 nm, or >650 nm with insignificant bleed-through to the other channels. This can be remedied by using multi laser systems.
6. For efficient sample preparation pre-conjugated antibodies can be used thus omitting the use of a secondary antibody for visualization. This reduces sample preparation time with 30-60 min and decreases cell loss due to the reduced number of wash steps. The drawback is that the pre-conjugated antibodies on the market are relatively few compared to un-conjugated alternatives.
7. The use of antibodies requires the cells to be fixed, permeabilized, treated with blocking solution and incubated with primary and secondary antibodies. Between all these steps the cells needs to be washed and centrifuged. In each step, cells are lost which might induce differences and errors in sample preparation for every step. The use of 96-well format can facilitate the sample preparation, but loss of cells would still be a major issue. Depending on the incubation times and protocol used sample preparation for staining an intracellular antigen is 1-4 hours.

In preparation of **Paper II,** we tried several methods on both the *Xenopus* and newly acquired mouse melan-a melanocytes and XB-2 keratinocytes. As expected, the cell trackers DiI and CMFDA were no good candidates; the 488 nm laser is suboptimal for excitation of DiI (20% of maximum) and it is not suitable for fixation. CMFDA has a broad emission peak that delivers a signal in both the FL1 channel (515-545 nm) and the FL2 channel (564-606 nm) and the signal is not well preserved after fixation. Antibodies against tyrosinase did react with melan-a melanosomes but not with *Xenopus* melanosomes. Although not evident in Western blots, XB-2 keratinocytes displayed a weak signal when using immunocytochemistry prompting further testing with blocking protocols to reduce the background signal. A PE-conjugated tyrosinase antibody was also tested, but had poor penetration characteristics compared to the un-conjugated. In order to optimize the signal a Qdot-conjugated secondary antibody was tested but it also suffered from poor penetration characteristics. Furthermore, the XB-2 keratinocytes did not test positive for the pan-reactive cytokeratin-antibodies we tested.

Even though these problems could be overcome with further testing of antibodies and fixation/permeabilisation and blocking protocols, the work of Lin et al. (2008) provided a solution to how a successful quantification could be made. By using a human ovarian teratoma transformed epidermal carcinoma cell line (HOTEC-C9) co-cultured with a human melanoic melanoma cell line (RPMI-7951) transfer was measured with flow cytometry. They used a FITC-conjugated anti-TRP-1 antibody for melanosome detection and a Cy5-conjugated anti cytokeratin 14 antibody for keratinocyte determination. The work by Lin et al. (2008) showed that the use of antibodies can be used for quantification and large amounts of melanin is however transferred when compared to our cell cultures. In 24 hours they found melanin transfer to approximately 20% of the keratinocytes, and after 72 hours almost 50 % of the keratinocytes were melanin positive. In addition, the transfer was stimulated by 100nM α-MSH, after 24 hours and about 70% of the epidermal carcinoma cells had taken up melanosomes. This is far more than we have been able to determine even after 86 hours of co-culturing in our *Xenopus* protocol. Even at these high numbers of transfer, the signal from the TRP1 positive keratinocytes is not clearly separated from the TRP1 negative population while the keratine 14 positive and negative populations are clearly separated. When taking into account the nature of the melanosome, it becomes apparent why it is hard to get a clear sub-population of TRP1 positive cells. The keratin signal depends on the amount of keratin in the keratinocyte and the main only interference is the presence of transferred melanin that may quench some of the signal. The keratin-negative melanocytes have large amounts of melanin that reduces any unspecific signal and increases the difference between the populations even further resulting in enhanced separation. The signal in TRP1 positive keratinocytes depends on 1. The amount of transferred melanosomes. 2. The amount of TRP1 on each melanosome. 3. The quenching by melanin that will reduce the signal. As the cells are spherical in shape when passing the detector it is possible that a cell with large amounts of melanosomes will have a smaller signal than a cell with a moderate amounts due to melanosomes near the incoming laser will shadow melanosomes deeper within the cell.

While the *Xenopus* system lacks suitable antibody markers to sort melanophores from fibroblasts, it was discovered that they are easily separated by their light-scattering properties. It was also possible to separate the mouse melan-a and XB-2 from each other, but fixation of the cells decreased the differences between the two cell types making it difficult to detect each cell type separately. Transfer of pigment does not alter scattering enough to be a marker for

melanin transfer. Scattering does make cell sorting easier and will only require a marker for transfer and no extra marker for cell type. The cells can not undergo fixation if scattering is used for sorting cell types.

When the Qdot-conjugated secondary antibodies were tested it became apparent that Qtracker® Cell labeling kits could be a useful tool for melanosome tracking. The kit contains the Qdots and a peptide to facilitate endocytosis. The Qdots are coated with the supplied peptide and upon incubation with melanophores they are taken up in the cells via endosomes and found to be incorporated into the melanosomes. After the melanophores were filled with Qdots, the labelled melanophores were washed and co-cultured with fibroblasts.

The use of Qdots was found to be superior to other available methods. The bleaching was minimal, the quantum yield high, and the Stokes shift (difference between exitation and emission wavelength) was large (>100 nm compared to 20-25 nm for traditional dyes). The emission peaks are relatively narrow, most are excitable by light <450nm. While custom filter sets for each Qdot are available, a simple UV excitation source and a long pass filter (such as Nikon UV-2A) worked perfectly when using immunofluorescense microscopy on any Qdot. Another advantage is the Qdots stay within the cells for several months thus allowing storage of samples for a long time, and they could also be re-analysed without fading. The Qdots are known to be unstable at low pH (below pH 4) which could result in degradation in acidic melanosomes. This can be balanced by membrane disrupting fixation of the cells in methanol and storage in neutral buffer solutions and no other negative effects were seen on Qdots fluorescence caused by the methanol fixation. Several different Qdots are available that can be used at different wavelength detections. This is also of great advantage since a second set of Qdots can be used for the recipient population (keratinocytes/fibroblasts). For example: Qdot 655 can be used for marking melanosomes and the signal would be detected in the FL3 channel (>650 nm), while Qdot605 can be used for marking of recipient cells and detection in the FL2 channel (564-606 nm) if a 488 nm laser is used for excitation.

Paper III: Roundup, pH and cytoskeleton integrity

Glyphosate-based herbicides such as Roundup are the most commonly used herbicides in the world. They are non-selective, able to affecting virtually all plant species, but reported to be relatively harmless to man and animals since it targets the plant-specific enzyme EPSPS (5-enolpyruvyl-shikimate-3-phophate). By introducing transgenic glyphosate resistant EPSPS variants into the commercial crop it is possible to use glyphosate to affect every plant in a field except for the transgenic species. This solution is very successful since it reduces the total amount of herbicides needed, while remaining highly effective at reducing unwanted weeds. At the same time it is a controversial solution as it requires the harvested crop to be transgenic.

When sprayed, excess glyphosate is considered to be easily bond to soil particles and degraded minimizing leakage into the groundwater and avoiding substantial bio accumulation. (Duke and Powels 2008). As the target for the herbicide is lacking in humans, glyphosate is not very toxic to humans and the effect of poisoning after drinking concentrated Roundup is possibly due to effects from the additives to Roundup such as the surfactant POEA (polyetylated tallow amine). The function of the surfactant is to increase the permeability of

the leaf cuticle, increasing glyphosate uptake into the plant. In order to increase the solubility of glyphosate it is often mixed into the herbicide solution as a salt with cationic agent such as isopropylamine.

Environmental concerns are that: 1. Transgenic species will spread and outcompete the natural species in the area. 2. Occurrence of Roundup resistent weeds due to mutation or gene transfer. 3. Bioaccumulation of glyphosate or degradation products thereof in the commercial crop are leading to negative health effects of the consumer, 4. Roundup can leak into aquatic environments due to low binding and degradation in the soil or improper use of the herbicide i.e. spraying close to bodies of water.

Amphibians seem to be easily affected by exposure to Roundup and several *in vitro* studies indicate negative effects on cell suvival (Benachour et al., 2009), hormone release (Hokanson et al., 2007), gene expression and mitochondria function (Peixoto 2005) at mM concentrations. The correlation between amphibian sensitivity and *in vitro* effects made it interesting to study the effects on intracellular transport of melanosomes in *Xenopus* melanophores. The aggregation and dispersion of the melanosomes in a confluent layer of melanophores in 96-well plates can easily be quantified by measuring the absorbance in each well after stimulation with an aggregating or dispersing agent. The process will be disturbed by any substance that interferes with receptors, signalling, motor activity or integrity of microtubules or actin filaments. The assay is practical for screening of effects of different substances at different concentrations. By combining aggregation assays with immunocytochemistry of the transport tracks microtubules and actin filaments (Aspengren et al., 2006) further information of toxic effects can be determined.

When testing the effects of Roundup, glyphosate, isopropylamine and glyphosate isopropylamine salt for 24 hours inhibition was seen on transport in the range of 0.5-5 mM. While these concentrations are in the same range as other *in vitro* studies we could observe a drastic colour change in the of the culture media at the highest concentrations indicating a change in pH. We decided therefore to measure the pH of the different dilutions and the effect of pH on transport. The melanosome transport was found to be pH dependent; shifting pH down to 6.5 decreased the transport while increasing pH to 8.5 enhanced the transport slightly. This indicated that some of the transport effects when treating the cells with glyphosate was due to the change in pH, since 1 mM concentration of either substance would cause similar changes in pH. The only exception was the glyphosateisopropylamine that affected the transport already at 0.5 mM where pH is still close to 7 in the medium.

The same dilutions were restested, but now also including samples where the stock solution was buffered to pH 7.5 to compare pH-independent effects. While the effect of isopropylamine was only slightly decreased showing significant difference only at 7 mM, the Roundup treatment showed decreased effect at 1.75 mM and higher concentrations. The inhibitory effect of glyphosate completely disappeared when using the buffered stock solution. One could therefore discuss whether the inhibitory effect on the transport by glyphosate is only a consequence of the low pH, or a combination of pH and glyphosate effect? As the glyphosateisopropylamine salt is inhibitory at low concentrations at near neutral pH, this indicates that the effect of glyphosate is not entirely pH-dependent. It could depend on effects of the different formulations as Roundup contains additives.

In an attempt to clarify these issues, cells were treated with a "high" or "low" concentration of the different substance either from an unbuffered stock solution or from a stock solution buffered to pH 7.5 and prepared for immunocytochemistry. Actin filaments and microtubules were present at low concentration of the unbuffered stock solution, but at the "high" concentration both actin filaments and microtubules were often disassembled. When using the buffered stock solution, the effects decreased in the Roundup treated cells, but the glyphosate treated cells looked similar to the controls. Interestingly, when melanophores were treated with a cell culture medium at pH 5.5, actin filaments did not disassemble but rearranged. They became numerous, thick and elongated. Microtubules often lost their radial arrangement and the melanosomes failed to aggregate to the cell center in response to melatonin treatment. The conclusion is that pH alters the cellular organization of cytoskeletal filaments, whereas high concentrations of glyphosate destroy them.

Glyphosate at high concentrations lowers the pH and at pH below 5.96 the molecule will bind one extra hydrogen and become a monovalent anion instead of a divalent (Chamberlain et al 1996). This increases the ability for the molecule to pass membranes allowing entry to the cytoplasm where glyphosate reverts to its divalent state. In the divalent state, glyphosate has an affinity for divalent cations. Binding to intracellular Ca^{2+} will interfere with signalling and enzymatic activities within the cell leading to an imbalance in actin and MT-assembly. In fact, hard water is an issue in agriculture use as it decreases the effectiveness of Roundup. Therefore chelating and acidic agents are often used in the spray solution in order to enhance the effectiveness of the herbicide. The difference in the pH effect between glyphosate and Roundup is probably caused by the presence of the surfactant POEA in the Roundup formulation, which increases the membrane permeability and allowing glyphosate entry into the cell, even at the divalent state.

The changing ability to cross membranes may explain the difference in toxicity; in large organisms glyphosate would have more opportunities to bind cations and be unable to cross barriers and accumulate in any cells as long as the pH is above 6 in the blood and tissue. In smaller aquatic organisms such as amphibian larvae, pH in the water would determine the ability for glyphosate to enter the cytoplasm and the situation would be worse in soft water.

The glyphosateisopropylamine salt was the most potent inhibitor in this study and had severe effect on cell adhesion suggesting an alternative mode of action. Similar studies (Benachour et al., 2009) show that glyphosate from high concentration formulas has more effect than low concentration formulas when diluted to the same concentration. In **Paper II**, the Roundup solution used is of a low concentration formula while the GI-salt solution is of a high starting concentration. Whether the extra effect is due to impurities or higher concentration of degradation products is unclear.

Paper IV: Photoreception and signal transduction in zebrafish melanophores

In **Paper IV,** we used inhibitors for Gq and Gs mediated signalling to determine the regulation of photo-induced dispersion in embryonal melanophores from zebrafish, *Danio rerio*. When these cells were kept in darkness melanosomes aggregated to the perinuclear region. When illuminated, the melanophores immediately reacted by dispersal of the melanosomes throughout the cell. This is very similar to the blanching of embryos reported in

both zebrafish and *Xenopus laevis* embryos at early development.(Logan et al., 2006). Treatment with inhibitors against PKA (H-89) and adenylate cyclase (SQ 22536) inhibited the dispersion thus indicating the signalling to be mediated by a Gs type of photoreceptor.

What is the reason for an early embryo to adapt to dark conditions by blanching and light conditions by darkening when this is the exact opposite of the reaction in adults or in late embryos at 120 hpf (Logan et al., 2006). The use of a photoreceptor on the early embryo melanophores would enable rapid regulation of aggregation and dispersion before the development of the more complex system of eyesight coupled to the brain followed by neuronal or hormonal signalling to the melanophores. One could speculate that the opposite directionality of the process compared to the adult might reflect a different set of needs during the early development. Studies of circadian rhythms show that cell division in zebrafish embryos peaks after dawn and it has been suggested that restriction of cell division during daylight would decrease the exposure of DNA to the UV radiation (Dekens et al., 2003) . By dispersion of the melanophores in daylight the embryo would obtain further protection against UV and this protection would be more useful than protection against predators via background adaptation at the early stage. Functional eyesight is established around 5 dpf simultaneously to the onset of adult type of background adaptation, and would allow visual information to reach the brain followed by subsequent regulation of the melanophores by neuronal and hormonal signalling. The observed photoreception must take place on the melanophore or via a neighboring cell signalling to the melanophore since removal of the head from the embryo also removes the possibility of reception via the eyes or the pineal and severes any neuronal connections. The evidence from similar studies of photoreception on Xenopus suggests the presence of an opsin on the melanophore itself (Daniolos et al., 1990, Ban et al., 2005).

In **Paper IV,** we could detect a relatively weak photoresponse in adult melanophores leaving us with the question about the role of photoreceptors in the adult fish. Light has although been found to affect melanophores in adult fish which can become tanned (Adashi et al. 2005) Red seabream lives normally at a depth of approximately 20 meters. When raised in cages in shallow water they become “suntanned” The tanning is due to both increased amounts of melanin and increased number of melanophores in the skin. This is in contrast to background adaptation where the normal response to long-term adaptation to light background results in decreased number of melanophores due to apoptosis (Sugimoto 2005). Although the mechanism for induction of apoptosis is well studied, no regulatory mechanism has been presented for the increase in cell number due to UV or bright light. If the embryonic photoreceptor is preserved in the adult, it could provide a model for regulation. One could speculate that melanophore photoreceptors are not significantly activated during moderate illumination and that the background and diurnal adaptation dominates via photoreceptors in the pineal gland and the eyes. The neuronal and hormone signals would be stronger than the cellular photoresponse by the melanophore, but during extreme conditions when they are exposed to high light intensity and/or at specific wavelengths, the “local” photoresponse would dominate over the signal for background adaptation. The animal would become darker in order to avoid damaging effects of UV. Such a system would have the benefit of activation prior to the occurrence of DNA damage in the skin which triggers the tanning respons in humans after the damage is already done (Yamagushi et al., 2007).

By inhibition of the photoresponse we conclude that the signalling pathway involved in zebrafish melanophores is mediated by signalling via a Gs-type receptor. The Gs and Gi type signalling with downstream increase/decrease in cAMP and PKA is well established as a regulator of melanosome dispersion and aggregation in zebrafish melanophores (Sheets et al., 2007; Richardson et al., 2008; Logan et al., 2006). These results were however surprising since photoreception in *Xenopus* via melanopsin is considered to be mediated by Gq (Isoldi et al., 2005). The initial study on the *Xenopus* cell line by Daniolos et al. (1990) suggested that the response was Gs mediated. This discrepancy may be a result of the different protocol used; in both studies he melanophores were aggregated by addition of melatonin prior to light exposure. Daniolos et al. (1990) removed the melatonin 1 hour before exposure to light (0.60 mW/cm^{-2}). In contrast Isoldi et al. (2005) retained a concentration of 10 nM melatonin in the media to keep the cell aggregated and the cAMPlevel low during exposure to predetermined non-saturating irradiance (2.082 mW/cm^{-2}). This light exposure dispersed the melanophores to about 50% of what is known as a complete dispersion achieved in the presence of 100 nM α-MSH. Amphibians have at least two melanopsin genes (Bellingham et al., 2006) and based on the results from the two mentioned studies they might differ in signalling pathways. While rhodopsin have been suggested as a second photoreceptor (Miyashita et al 2001) no functional studies have been done. Neither of the two studies found any relation to cGMP signalling in the photo-response thus offering no support for a functional role of rhodopsin. Later studies with mouse and human melanopsin have detected the involvement of Ca^{2+} in signal transduction further supporting the Gq type signal (Giesbers et al., 2008; Kumbalasiri et al., 2007). In Xenopus melanophores the Ca^{2+} signalling is thought to increase the activity of PP2B which inhibits adenylate cyclase thereby diminishing cAMP levels induced by stimulation of α-MSH (Isoldi et al., 2009). This enables the melanopsin to not only induce dispersion via PKC, but also to inhibit cAMP mediated dispersion and possibly induce detachment of myosin Va from actin filaments (Nguyen and Higuchi, 2005) in subcellular regions where sufficient Ca^{2+} concentration can be obtained.

Teleost melanophores seem to use another receptor-signalling pathway than amphibians. They are known to aggregate upon increase in IP_3 (Fujii et al., 1991) and PKC (Abrao et al., 1991) is involved in downstream signalling after activation of Gq. This suggest the light receptor is not coupled to Gq-signalling, because then the response to light would result in aggregation rather than dispersion Light-induced signalling has been shown in erythrophores *Nile tilapia* (Ban et al., 2005); they possess a red opsin signalling via Gs, and a green opsin signalling via Gi, enabling the erythrophore to aggregate or disperse their pigment in response to light at different wavelengths. A Gs-type of signalling has also been described in a green-sensitive opsin in the eye of box jellyfish (Koynanagi et al., 2008).

In **Paper IV** we used RT-PCR and immunohistochemistry for identification of the receptor. The only well studied photoreceptor in melanophores is *Xenopus laevis* melanopsin that was discovered in 1998 (Provencio et al., 1998) and although rhodopsin has been implicated (Miyashita et al 2001). The diversity of melanopsins in the zebrafish genome, opn4a, opn4b, opn4l, could provide a mechanism for differentiation of intracellular signalling. Melanopsins are expressed in the teleost eyes and brain (Drivenes et al., 2003) so by isolation of the heads a positive control for primers could be obtained. Both rhodopsin, opn4a and opn4l mRNA were detected in the head preparations of 5 dpf embryos but not in the 2 dpf embryos. By using an antibody against human melanopsin, where the target region is 82% and 78% identical to opn4l and op4l respectively, we could detect melanopsin expression in RPE cells

in 5 dpf, but not 2 dpf embryos which is in agreement with the expression data. We have not yet identified the exact identity of the RPE melanopsin, but this is to our knowledge the first detection of melanopsin in the teleost eye by melanopsin specific antibody. The ability to use an antibody opens up many possibilities for future studies of these interesting receptors. Melanopsin have previously been detected in the mouse retina (Peirson et al., 2004). Although the function in the mammalian RPE is unclear, zebrafish RPE cells have a circadian variation in melanosome positioning (Menger et al., 2005). During daylight, the melanosomes are dispersed into the RPE-cell dentrites that infiltrate the visual cell layer thus protecting them from damaging from illumination. By night, the melanosomes retract from the RPE dendrites leaving the visual cells unprotected, but with an increased capacity for light detection. The function may be similar to that of the mammalian papillary reflex which is also regulated by melanopsin in the retinal ganglion cells (Lucas et al. 2003).

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

One of the aims for this thesis was to learn more about how melanophores transfer melanosomes to other cells for long-term colour change. A cell line from *Xenopus laevis* was used which has the advantage that melanophores and skin fibroblasts can be cultured together in the same cell culture medium. The transfer quantification in the first study was made manually which was time-consuming. Several different attempts were made to improve the manual method, but it was not until fluorescent semiconducting nanocrystals were used in combination with flow cytometry that a promising new method was introduced. The nanocrystals do not only fluoresce they can also be detected by electron microscopy which can be used for future detailed analysis of melanosome transfer. It will furthermore be of interest to improve the method for culturing of mouse and human melanocytes, where quantification of melanosome transfer is of interest both medical and cosmetic compounds that can affect transfer.

Another aim was to continue and develop the use of the melanophore aggregation assay as a model system for toxicological studies. It has several advantages; it is rapid and effects on intracellular transport can easily be detected by spectrophotometry. However, this assay can only be used as a first test of different chemicals, as the quantitative results are affected by effects on the morphology and localisation of microtubules and actin filaments. By combining the aggregation assay with immunocytochemistry a much more detailed analysis can be achieved. Microtubules and actin filaments are of outmost importance for cellular functions such as intracellular transport, mitosis, sperm movement and muscle function. The system could therefore be of interest for performing toxicological data and risk assessment.

In spite of many efforts to find conditions to establish a zebrafish, *Danio rerio,* melanophore cell line there was no success. However, during the work, a photoreceptor was found in zebrafish melanophores, as well as in the zebrafish retinal pigment cells in the eyes. There are several interesting possibilites for future studies of the characteristics and function of photoreception and melanopsins. We are currently implementing microinjection techniques with the ambition of creating morpholino-based gene knockdowns in the zebrafish embryo. As the eye provides an excellent positive control it is possible to adapt *in situ* hybridisation for detection of specific melanopsin mRNA within melanophores. Melanopsin has been detected in the tail of *Xenopus* tadpoles, but in zebrafish, the expression appears too low for reliable detection. We have tried during several years to produce a zebrafish melanophore without success. Another alternative to get a crude preparation of melanophores could be made with the use of Laser Microdissection and Pressure Catapulting (LMPC) techniques and the samples could then be used for PCR.

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