Activity and Crosstalk of STAT3 and BMP Signalling Pathways in Pluripotency Control of Mouse and Medaka Stem Cells

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1. Introduction

Cells of all organisms communicate with their environment in a multitude of ways. This communication is especially elaborate in multicellular organisms, where cells not only sense outside cues, but also those sent out by many and more sister-cells building the same body. Hundreds of different sensors are distributed over a cell, connected to an extremely complex signal transduction system. All the thus received information are then integrated and converted to cellular responses.

Even though signal transduction pathways are among the most scrutinized topics in molecular biology, there is still little understanding of the interwoven crosstalk within and between thus far isolated networks. Thus far, linear transduction systems are well known and accepted. The interaction between pathways, the resulting signal integration at connecting molecular nodes and its outcome, including their cellular interpretation are rarely reported in detail and much less understood.

To look at the interaction between signal transduction systems, two pathways were chosen that met the following criteria:

(1) They should be well studied in their basic transduction mechanisms.

This is obviously important as studying interaction of pathways with unknown signal transduction mechanisms is hardly possible. Consequently, the more information on the single, isolated pathways are available, the broader the experimental spectrum for crosstalk analyses will be.

(2) The literature should give hints at a systemic interaction between the pathways.

Quite often there are phenotypic consequences reported for situations where three experimental outcomes follow treatment with ligand A, ligand B and ligand A+B. This already hints at a molecular integration of the given signals, finally leading to the observed phenotypes. Even better scores were given to such examples where the signal transducing proteins of the different pathways were shown to physically interact under certain circumstances.

(3) They should be important for an *in vivo* and an *in vitro* system.

During cultivation procedures the physiology of cells may change rather drastically; probably as a direct result of both new additional and also lacking signals from the environment. While investigating a signalling network only present in-vitro could still yield basic information and present a good number of experimental approaches, a network not present in culture but only *in vivo* should be considered more relevant, yet technically hard to study. Obviously, a situation where signal crosstalk plays an important role *in vivo*, but is also replicable in various in-vitro systems, features both: variable experimental approach combined with interesting and directly relevant biology.

(4) They should use shuttling transcription factors as transduction vehicles.

This feature would make experiments a lot easier in many ways. First of all, latent transcription factors are generally thought to be easy to tag and consequently to track, both live – when fused to a fluorescent protein – or in biochemical assays – when fused to purification sequences such as FLAG, MYC or HA. Secondly, ectopic overexpression of most functional proteins will certainly disturb cellular responses. In the case of shuttling transcription factors, this problem probably exists, but is less serious. These proteins will not trigger target gene expression without being activated by a signal given by their respective activators . A dormant, and cytoplasmically localised transcription factor is hence less prone to unwanted ectopic effects. And finally, these factors are rather easy to interpret, as their cellular localisation usually hints at their activity level – as there is no target DNA site, transcriptional activation will not occur in the cytoplasm. It should be noted though, that functions other than transcriptional regulation at specific DNA sites can not be as easily assessed.

Prime examples meeting these criteria are the Bone Morphogenetic Protein (BMP) and the Leukaemia Inhibitory Factor (LIF) pathways. Both are well characterised with respect to their linear transduction mechanics (f*igure 1, figure 2*), they are important for pluripotency control in embryonic stem cell culture and for early development *in vivo*, they have been shown to counter-regulate genes in various differentiation systems and both employ latent transcription factors for signal conveyance (Smads for BMP and STAT3 for LIF).

1.1 The BMP pathway

1.1.1 One signal, many consequences

BMP signals affect many different kinds of cells. The most defining results of BMP signal transduction are seen in stem and progenitor cells. Fascinatingly, BMP exposure is far from producing a fixed outcome for receiving cells: While mesodermal stem cells will react to certain levels of BMP2/4 by differentiating into osteoblasts or chondrocytes [1], one step earlier they will be pre-defined during gastrulation in their identity along the anterior-posterior axis of the embryo. Neural stem cells will become astrocytes [2] when exposed to high levels of BMP2/4, while hair-progenitors of the inner ear will go into apoptosis [3] – this difference being even more pronounced due to the common neuro-ectodermal origin of both progenitors. Primordial germ cells will first be defined in their lineage and later be subject to proliferation control through BMP signalling.

All these different outcomes appear to be defined by several factors. First and foremost, the molecular make-up of the receiving cell is obviously important. Then, however, the cell`s transduction machinery is engaged by different molecular cocktails depending on the environment. These are usually referred to as niches and have recently gained more attention as researchers begin to understand the importance of re-creating or re-defining niches to be able to guide stem-cell behaviour in-vitro.

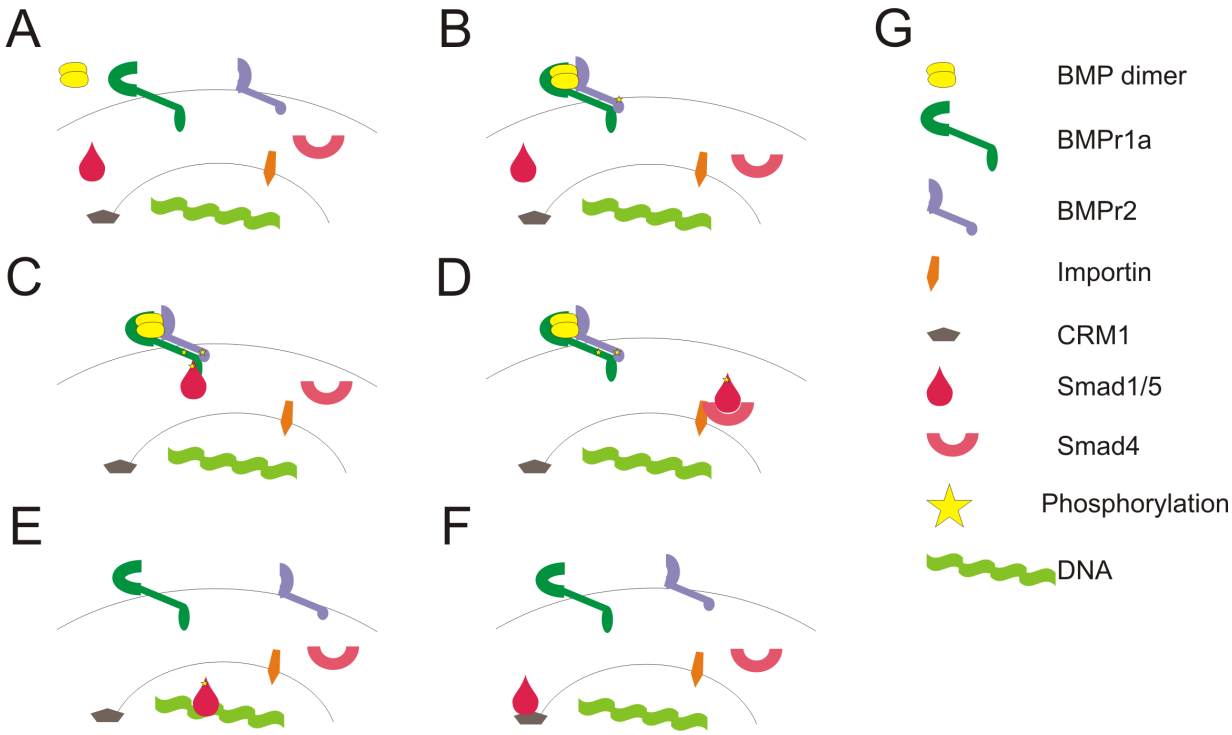

Figure 1: The classical BMP pathway. The cartoons (A) to (F) depict the common mode of signal transduction used by BMP2/4 ligands. Upon binding to a type I/II receptor heterodimer, a phosphorylation cascade is initiated that finally targets the C-terminus of Smad1/5. The phosphorylated Smad proteins are able to multimerise with Smad4 and consequently accumulate in the cell nucleus where they influence transciption of target genes. (G) show the legend for this scheme.

1.1.2 BMP signal modulation

The above mentioned examples of different BMP signal responses also represent different modes of signal modulation.

First, in the extracellular space, BMPs are modulated by (homo- and also hetero-) dimerisation [4][5]. Then, there are at least seven different BMPs, many engaging overlapping receptor types. Additionally, there are several different BMP decoys that bind and thereby mask BMP dimers (Noggin, Chordin and Follistatin).

Then, at the cell membrane, a variety of promiscuous receptors are potentially able to bind BMP ligand dimers. In the classical model, one type I receptor binds a ligand, changes its confirmation as a result and subsequently recruits a type II receptor into the complex [6]. The type II receptor is then phosphorylating the type I receptor, enabling subsequent binding of Smad proteins to the phsophorylated docking site, where they are in turn

phosphorylated by the type I receptor's serine-threonine kinase domain. This is, however, a by far simplified view as demonstrated by recent publications [7] that link pre-formed receptor multimers to different signal transduction routes and outcomes. By now, it has been demonstrated that successive recruitment of type I and II receptors will actually lead to signal transduction via the p38 mitogen activated protein kinase (MAPK) pathways rather than through Smads. For this, a series of adapter proteins including Tab1, transforming growth factor ß activating kinase 1/2 (Tak1/2) and XIAP [8] are needed. Preformed heteromeric receptor complexes will, however, activate Smads upon BMP ligand engagement. Then, there are also pseudo-receptors, e.g. BAMBI [9]. These block signal induction by sequestering ligands at the cell membrane. Further downstream, BMP receptors can associate with different Smad proteins and also with a wide variety of adaptor proteins, which all influence signal progression. *Table 1* lists the seven known mammalian Smads and their classical role. It should be noted that these Smads are not just regulated by differential expression, but also by secondary protein modification in the form of sumoylation, ubiquitinylation and of course phosphorylation . Additionally, the decoy-Smad7 acts as dominant negative molecule by binding to type I receptors, thus limiting access of other Smads. Smad6 is a competitor for already activated Smads as it binds and sequesters Smad4, which is needed by all phospho-activated Smads for import into the nucleus [10].

Name	Type	Ligands	Receptors (type I)	Function
Smad1	R-Smad	AMH, BMP2/4/7	ALK1/2/3/6	Transcriptional regulation
Smad2	R-Smad	AMH, BMP2/4/7	ALK1/2/3/6	Transcriptional regulation
Smad3	R-Smad	Activin, Nodal, TGF-b	ALK4/5/7	Transcriptional regulation
Smad4	Co-Smad	All	-	Co-Smad needed for All R-Smads
Smad5	R-Smad	AMH, BMP2/4/7	ALK1/2/3/6	Transcriptional regulation
Smad6	I-Smad	-	All	Decoy Smad, inhibition of Smad interactions
Smad7	I-Smad	-	All	Decoy Smad, inhibition of Smad interactions
Smad8	R-Smad	AMH, BMP2/4/7	ALK1/2/3/6	Transcriptional regulation

Table 1: An overview of Smad proteins and their functional relations.

While Smad's movement rates into and out of the nucleus are again regulated by so far hardly understood mechanisms, the nuclear activity of Smads is as well subject to a better known plethora of modulation mechanisms. First of all, there is the level of protein-protein

interaction in transcriptional complexes. Association of Smads with chromatin modifying enzymes (e.g. P300 [11]) and with other transcription factors (e.g. STAT3 [12]) multiply potential target gene numbers. De-phosphorylation of Smads at different regulatory sites needed for DNA binding, protein-protein interaction, nuclear retention and transcriptional activation are further enhancing the signal outcome variability.

1.1.3 BMP target genes

One of the best characterised target genes of classical BMP signalling via Smad1/Smad4 are the Inhibitors of DNA Bindung (ID) genes. A Smad1/Smad4 complex preferentially binds to GC-rich elements in combination with sequences named Smad-Binding-Elements (SBEs, sequence: GTCT). These have been found and characterised in the promoter of ID1 [13]. Transcriptional upregulation of ID1 and ID2 occurs shortly after nuclear Smad1/5 accumulation and is thus far considered to be the first transcriptional readout for complete BMP signal transduction. The family of ID proteins is generally seen as repressory proteins that bind to basic Helix-Loop-Helix (bHLH) factors and thus inhibit the DNA binding ability of certain transcription factors. In this way, IDs are able to effectively block target gene transcription of a wide variety of transcription factors such as NeuoD, Ngn and MyoD. Hereby, the differentiation initiation by these proteins is blocked. At the same time, IDs are able to inhibit repressing effects of transcription factors and thus enable up-regulation of other genes [14].

1.1.4 BMP signalling during early development

Interestingly, BMPs were not initially identified by mutational screens of Drosophila, Xenopus or zebrafish. As their name suggests, these growth-factors were identified by protein isolation from bovine bone (more than 1 ton was needed), because they retained their osteoinductive effect. Wozney et al. [15] were thus able to clone the first mammalian morphogens. As mentioned above, BMPs do much more than just stimulate bone growth. Their role in early development of vertebrate embryos was found mostly by genetic screens and targeted deletion in mice. In BMP4-null mice, mesoderm induction does not take place, making BMP4 indispensable for correct gastrulation [16]. The same is true for BMPR1a, the receptor for BMP2/4 [17]. Knock-out mice for this locus show normal pre-implantation development, but strong abnormalities including a lack of mesodermal differentiation become apparent between E7.0 and E9. In both, the ligand and the receptor deletion mice, epiblast proliferation is strongly defective as well, suggesting not only inductive effects, but also cell-cycle stimulatory influences of the signalling pathway.

Studies in Xenopus and Zebrafish established the dorso-ventral axis patterning role for BMPs and their antagonists. While the exact players vary slightly between species, the general idea is as follows: BMP proteins show strong ventralising activity as evidenced by loss-of-function mutations and assays (e.g. the zebrafish *swirl* mutant affecting Bmp2b [18]). The expression of BMP ligands usually excludes the organizer/shield/node regions of the early gastrulating embryos. Conversely, the ligand-antagonists Chordin, Noggin and Follistatin are expressed within those patterning centers [19]. While all of these factors are secreted proteins, it has not yet been possible to localise them on protein level. However, there is strong indirect experimental evidence that predict opposing protein gradients of both agonists and antagonists that eventually lead to the establishment of a signal-strength gradient along the thus forming dorso-ventral axis.

1.2 The JAK-STAT pathway

1.2.1 Basic principles

Several different ligands including interleukins (IL), Leukemia Inhibitory Factor (LIF), granulocyte stimulating factor (GSF), ciliary neurotrophic factor (CNF), alpha- and gamma interferon (IFN), epidermal growth factor (EGF) and many others are able to use a combination of a specific receptors (e.g. LIF-receptor for LIF) heterodimerising with the glycoprotein-130 (gp130) receptor. Associated with the intracellular parts of these receptors are often Janus Kinases (JAKs) that are phosphorylated by gp130 upon its ligand-induced structural rearrangement. After being activated themselves, the JAKs phosphorylate the receptors which then remodel and present a binding site for Src homology 2-domains (SH2) [20]. The signal-transducer-and-activator-of-transcription (STAT) family of latent transcription factors contain such a domain and bind to Y-X-X-Q motives as monomers[21]. Once they are bound, they themselves become phosphorylated. In a simplistic model, this phosphorylation will lead to dissociation of the given STAT from the receptor complex, upon which they are free to homo- or heterodimerise. The dimerisation enables their importin-alpha dependent nuclear import[22][23]. Only as dimers are STATs able to bind DNA and act – in most cases – as positive regulators of transcription (*figure 2*).

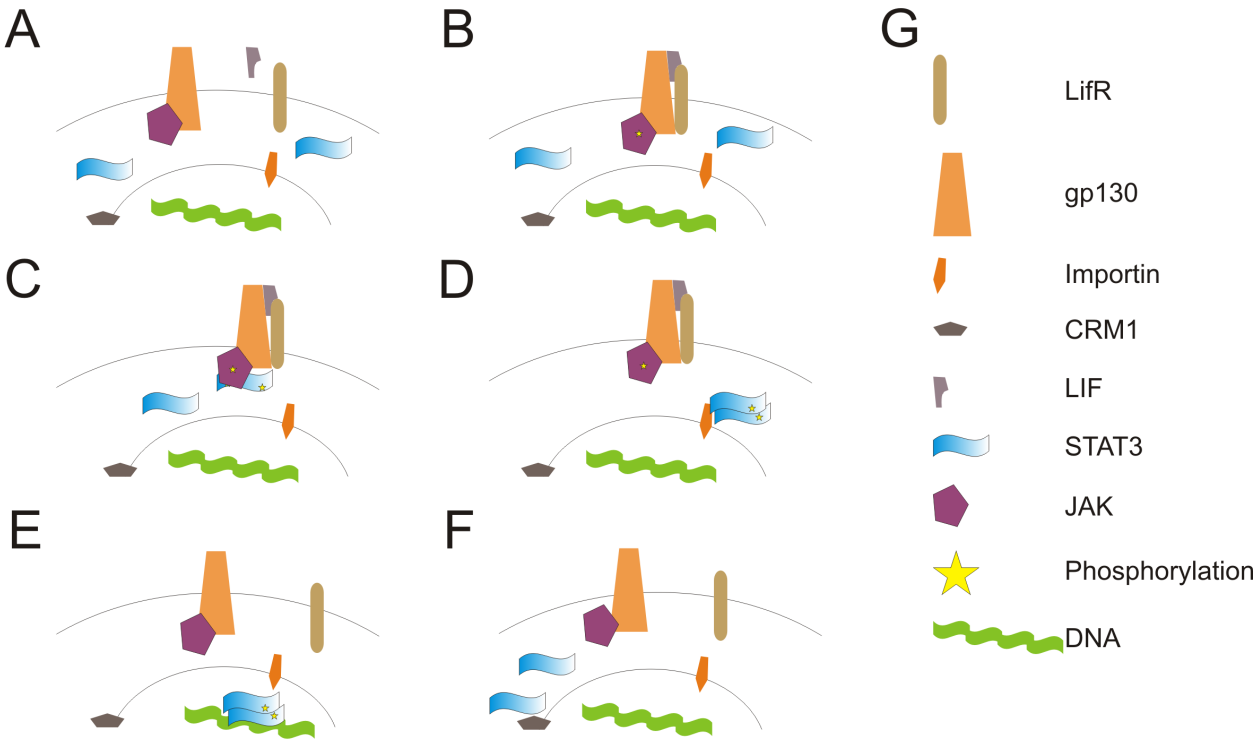

Figure 2: The classical LIF-STAT3 pathway. This scheme shows an oversimplified signal transduction cascade from LIF binding (A), over receptor heterodimerisation and JanusKinase activation (B), to STAT3 phosphorylation (C), consequent homodimerisation (D)and import into the nucleus (E), where the the dimers accumulate and bind to DNA, usually activating transcription of target genes, before they are exported and dephopshorylated again (F). (G) lists the symbols for the sketched molecules.

1.2.2 STAT3 modulation

The real situation is quite more complicated and far from unanimously accepted. Several studies dealing with the mobility of STAT3 in different states quite clearly prove that STAT3 is well able to translocate to the nucleus in a monomeric, unphosphorylated from [24][25]. As a common principle for regulation of latent transcription factors such as Smads and STAT3, their nuclear-cytoplasmic shuttling is a key feature.

Higher order regulation of signal transduction, gene expression, RNA localisation and other regulatory mechanisms of eucarytotic cells are only possible due to a selective barrier between cytoplasm and DNA, the nuclear envelope. There are three generally accepted ways of passing this nuclear membrane [26]:

(1) Below 40kD of size, the nuclear-pore-compexes (NPCs) are large enough to allow

passive diffusion of proteins. Of course, small molecules such as ions etc. are also able to simply pass the envelope without a need for active transport.

(2) Larger proteins are sometimes able to directly bind to NPC-proteins (the beta-karyopherins, including importins and exportins) and activate their GTP-dependent passage machinery. Whether this is at all possible for signal transducer proteins such as STATs and Smads is not clear yet.

(3) Finally, proteins that are not able to directly attach to the NPC transporters often use nuclear-localisation signals (NLS) – a quite variable stretch of basic aminoacids – which is then recognised by adapter proteins of the importin-alpha family. These then regulate the transport by bridging the cargo-protein (with the NLS) and the beta-karyopherin importin-ß1. NLS sequences are often conditional, hidden on the inside of proteins, and only become bindable after conformational changes often induced by protein modifications such as phosphorylation. In the case of STAT3 however, the NLS located within the coiled-coil domain is a constitutive one. Consequently, STAT3 monomers are able to associate with importins alpha-3, -5 and -7 [27][22][23] and constantly trigger nuclear import – even when not activated through signal transduction.

Once inside the nucleus, STAT3 can finally make use of its DNA binding domain and the C-terminal transactivation domain (SH2, *figure 3*). DNA binding of so-called GAS sites is only seen in the dimeric form [28].

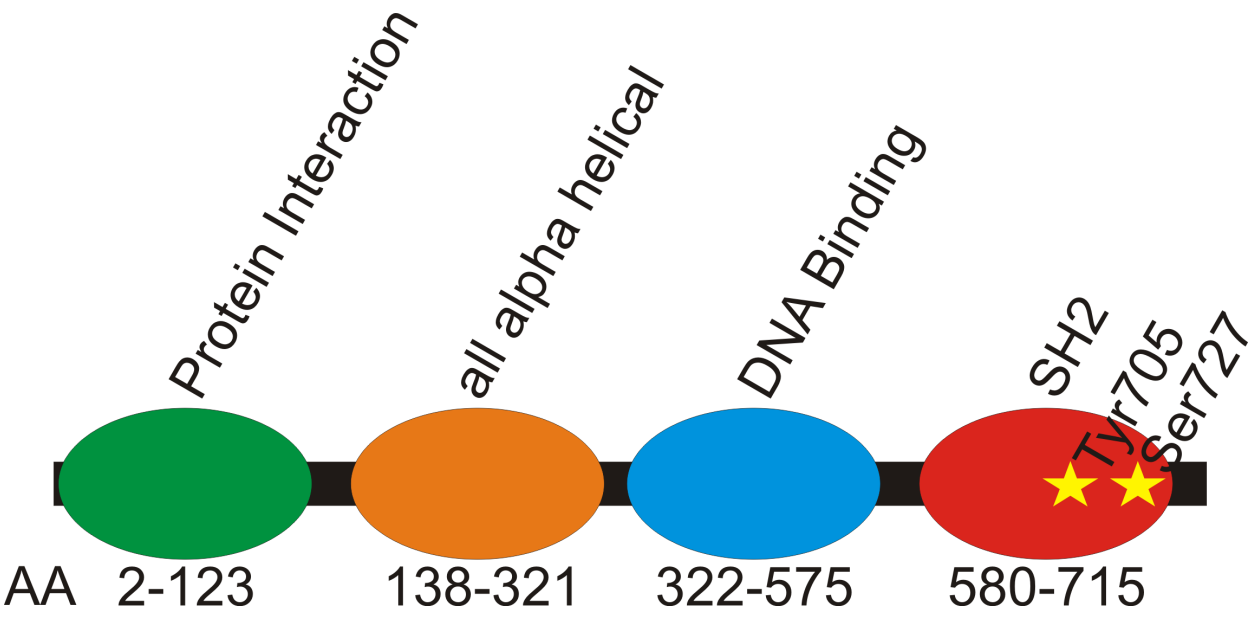

Figure 3: Domain structure of STAT3. Four distinct domains are conserved between all STAT proteins. In the last domain, two conserved residues that are used for functionally influencing secondary modifications are highlighted by yellow stars.

Studies focusing on the mobility of STAT3 have uncovered that pSTAT3 accumulates within the nucleus, due to a simultaneous increase of nuclear import accompanied by a decrease of nuclear export [29]. One explanation for this phenomenon might be a stabilisation and consequently lower mobility of pSTAT3-dimers bound to DNA when compared to the free, monomeric form. Matching this theory, are data by Herrmann et al. [30]. Not only were they able to correlate Serine727-phosphorylation of STAT3 with accumulation of the p705p727-STAT3 (ppSTAT3) protein at nuclear-body like sites at the periphery of splicing-factor compartments, but they could also distinguish two differently mobile subgroups.

This DNA-binding and subsequent transcriptional activation have long been seen as the sole function of nuclear pSTAT3 in either homodimers or STAT1-STAT3 heterodimers. But, recent publications convincingly show that monomeric, non-phosphorylated STAT3 may indeed influence transcription of several genes [31]. Likewise, transactivation by STAT1 has also been suggested when the monomeric form associates with enhanceosomes [32]. This, together with the fact that a Fluorescence-Resonance-Energy-Transfer (FRET) is readily detectable between YFP/CFP tagged, inactive STAT3 (through mutation Y705F non-phosphorylatable) forms, as well as the presence of about 25% of STAT3 proteins within the nucleus in the monomeric state [33], strongly support the idea of alternative functions for STAT3 apart from strict phosphorylation-activated DNA binding.

Obviously, an important part of signal transduction is the regulation of its termination. Only recently Zhang et al. [34] were able to identify the Receptor Protein Tyrosine Phosphatase T (PTPRT) as the first enzyme able to de-phosphorylate Tyr705 of pSTAT3. Consostently, PTPRT is the most commonly mutated PTP in human cancers, and constitutively activated STAT3 is often associated with tumour formation.

Export of both, the monomeric and dimeric forms of STAT3 are probably mediated by the nuclear-export receptor Chromosome Region Maintenance 1 (CRM1). This is evidenced by studies using small molecules to inhibit CRM1 function, which results in nuclear accumulation of STAT3 [35]. Even though in this study three potential nuclear export signals (NESs) within the STAT3 aminoacid sequence were identified and functionally verified by fusion to GFP, the crystal-structure of STAT3 dimers bound to DNA [28] shows these protein-streches burried inaccessibly. While this makes sense as DNA-bound STAT3 has a much lower mobility than the monomeric form, the existence of CRM1 independent export mechanisms is a viable alternative. This notion is further backed by the finding that the CRM1 inhibitor Leptomycin B is not able to completely block STAT3 export [36].

1.2.3 STAT3 as a mediator of signalling crosstalk

As mentioned above, STAT3 is not solely influenced through phosphorylation at Tyrosine705, but also by control of the phosphorylation state of Serine727. This residue seems to be responsible for about 70% of the transcriptional activation ability, demonstrated by a likewise drop in promoter upregulation upon point-mutation. JAKs are, however, tyrosine kinases. Hence, other enzymes must be responsible for this level of regulation, and indeed Tian et al. and others were able to show that the MAPK ERK1/2 is able to drive phosphorylation of STAT3 at Ser727[37][38]. Immediately, this attributes STAT3 the role of a signal integrator with a wide range of inputs from two sides: the MAPK upstream pathways [39][40] and the aforementioned IL-like group of cytokines.

Another link of signal transduction pathways through STAT3 is indicated by the integration of BMP signalling during development of neuroprogenitor cells: The fate of fetal neuroepithelial cells is subject to growth factor regulation. Upon simultaneous stimulation with LIF and BMP strong induction of Glial-Fibrillar-Acidic-Protein (GFAP) goes along with differentiation of the progenitors into the astrocyte lineage. In 1999, this synergistic effect was found to be a result of the co-binding of Smad1 and STAT3 to the nuclear integrator protein p300, and only this complex is able to induce the GFAP expression to a sufficient degree to initialise differentiation [12]. This was the first demonstration that the BMP

pathway and the JAK-STAT3 pathway intersect – at least within a transcriptional activator complex.

In combination with other work that analysed p300 function in more detail , one mechanism of signal modulation was caught in action as well: competition for chromatin remodelling factors such as p300 and CBP[41]. It appears as if those modifiers are in short supply within most cells, while the signal transducers and other transcription factors are present excessively. In many cases – especially in those where new cellular programmes such as the initiation of differentiation are the subject of signalling – chromatin state changes are needed for the transcription factors to be able to do their job. Hence, the ability to re-program a cell fate is often not directly dependent on those transcription factors, but on their ability to out-compete others for attracting chromatin remodelling complexes.

1.3 Signalling in Stem Cells

In order to find and understand signal transduction networks rather than pathways, model systems with a high degree of flexibility in the form of receptors and responses are necessary. Prime examples for such models are stem cells. They are usually able to receive a wide variety of signals and often interpret them as directions for complex cellular responses that manifest in differentiation.

Another advantage of using different stem cell systems lies in the power of comparative analyses between graded levels of differentiation potential. Only the fertilized egg (and early cleavage cells) can be attributed totipotency, which is the ability to initiate embryonic development and form any cell type needed for complete development. The in-vitro cultivatable embryonic stem cells are more restricted in their fate choice, hence considered pluripotent; able to become any cell type of the body-proper, but not able to initiate development of an embryo upon implantation. Usually, embryonic cells after the first cleavages are able to give rise to such stem cell lines in culture. Later in development, and partially still persisting during adulthood are the so-called multipotential stem cells. These include a wide variety of cells that have not, unlike most of the other cell types of the body, arrested their cell cycle. Lastly, the unipotent stem cells [42] are direct progenitors of one specific cell type and are able to either multiply themselves through division or terminally differentiate.

One hallmark of stem cells is their ability to divide in a probably unrestricted fashion and produce two daughter cells with the same potential, thereby enlarging the pool of stem cells. The just described symmetric stem cell division is but one form of cell-cycle possible for stem cells, but current concepts [43] include at least two more.

A second type is the asymmetric cell-division, which in most cases leads to two very different daughter cells. One that will rather soon differentiate into a more restricted or even terminally differentiated cell, and another cell that remains a stem cell just like the mother cell.

The third type of cell-division is again symmetric, but will lead to two daughter cells with less differentiation potential compared to the mother cell, both usually also undergoing terminal differentiation.

Among the best studied multipotential adult stem cells are the haematopoetic stem cells [44]. They produce a rather broad spectrum of different cell types after division and they are also able to give rise to enormous amounts of daughter cells, while remaining

undifferentiated themselves. Other stem cells such as muscle satellite cells or neural stem cells divide rather reluctantly and only give rise to one or two different cell types.

1.4 ES-cell pluripotency and BMP/STAT3 crosstalk

Since 1988, the key player in ES-cell media for the best established culture system, murine ES-cells, has been LIF[45][46]. Even though cultures have to be additionally supplemented by serum, feeder cells and other factors, LIF is considered to be necessary to maintain pluripotency. The signalling cascade triggered by LIF is transduced through phosphorylation and subsequent translocation of STAT3 to the cell nucleus [47][48]. It should, however, be noted that recent studies in nonhuman primate ES-cell cultures [49] as well as in human ES-cell culture systems [50], have demonstrated complete independence of LIF and STAT3. In the mouse system, feeder cells and serum can be omitted if BMP2/4 and LIF are present in the medium [51][52], resulting in a very defined two-factor system to study pluripotency. The same work demonstrated that the downstream target genes primarily responsible for the pluripotency maintenance effect of BMPs under these conditions are the ID genes. ID gene expression is further enhanced by another well known pluripotency associated factor, called Nanog [53], in a not yet understood way.

Adding to the picture are data obtained by microarray-based analysis [54] of murine stem cells, in which several genes with STAT3 dependent expression were identified. Among the list of strongly up-regulated genes after LIF/STAT3 inactivation are factors generally associated with TGF-b and BMP signalling cascades including Lefty1, ID1 and ID2. These data already indicate negative transcriptional regulation between LIF/STAT3 signalling on one hand, and TGF-b/BMP on the other hand. Strong activation of the BMP pathway was shown to lead to differentiation of embryonic stem cells into mesodermal and endodermal lineages, whereas neural differentiation is actively suppressed [55]. Low levels of BMP, followed by transduction via Smad1, already leads to up-regulation of markers typical for early stages of mesodermal differentiation including the transcription factor Brachyury. Present in only low amounts, Brachyury forms a complex with STAT3. This complex has been shown to bind to the Nanog promoter and enhance its transcription. Nanog protein in turn was shown to bind to Smad1 and thus to suppress formation of Smad1 transcriptional activator complexes [56]. Closing the regulatory circle, a prime target of the thus inhibited Smad1-complex mediated transcriptional activation would be *brachyury*. Thus, early stages of differentiation triggered by low level BMP signalling are reversible by simultaneous presence and action of Nanog and STAT3. Consequently, support of pluripotency by BMP

signals is not only highly dose-dependent, but also needs to be counter-regulated (e.g. by STAT3 and Nanog). In summary, the BMP-signalling pathway promotes pluripotency only indirectly by driving expression of ID genes in a Smad1-dependent manner. ID-proteins block neural differentiation of ES-cells by sequestering transcription factors needed to initiate commitment to this lineage. Concurrently, the differentiation induction effects of BMP are counteracted by STAT3 and Nanog, which are able to suppress activation of Smad1-target genes necessary for differentiation into mesodermal and endodermal cell fates. In other words, the essence of defined medium murine ES-cell culture appears to be the simultaneous action of STAT3 and Smad1 in a balanced ratio. Downstream, negative regulation of differentiation programmes for mesodermal and endodermal fates (mediated by STAT3) as well as neuro-ectodermal lineages (controlled by IDs) is initiated, resulting in the blockage of any kind of differentiation (*figure 4*).

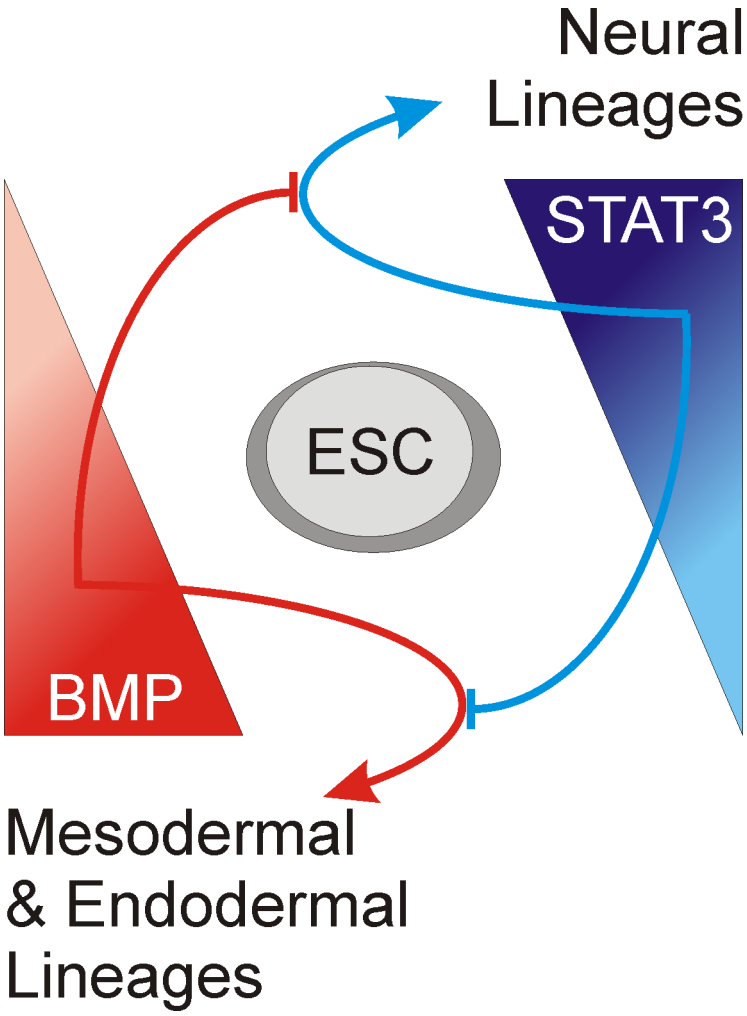

Figure 4: Counterinhibition of STAT3 and BMP signalling in mouse ES-cells. The diagram shows a simplified model suggested by Ying et al. for the control of pluripotency in mouse ES-cells. Without other influencing factors, the cytokine LIF and the morphogen BMP are able to block the differentiation inducing effects of the each other, thus keeping the stem cells in an undiffertiated state.

1.5 Aim of this thesis

The main focus of this work was the functional elucidation of signal integration between the STAT3 and the BMP2 pathway. Hence, the major signal transducer proteins of both pathways of Medaka were cloned and visualised both live, as fluorescence-protein fusions, and by immunofluorescence . As both are latent transcription factors that are only able to act as such after secondary modifications in the form of phosphorylation, two independent features were assessed to describe their status: phosphorylation by Western blot – only possible for STAT3 – and subcellular localisation. The availability of embryonic stem cell cultures from both, mouse and Medaka was used in a comparative approach of their molecular features in respect to these two pathways. Already known roles and functions for both pathways in the mouse ES-cell system were compared to data gathered from the in this respect so far little investigated Mes1 cells. Such a functional comparison is aimed at revealing a common basis of stemness control.

In parallel, a second level of comparison, between the Mes1 cell culture system and its embryonic origin, the blastula embryo, offers further insights into the relevance of artificial tools in studying biological processes in the field of stem cells. *In vivo*, stem cells are often found in highly specific environments, so-called nieches. To date, the growth conditions of stem cells in culture are hardly comparable to the molecular cocktail the cells originated from. One way to adding more knowledge in that area is the analysis of signalling pathways *in vivo*. A lot of data is available on the earliest patterning and differentiation events happening in the embryo. Especially the BMP pathway is extensively involved in a wide spectrum of cell fate decisions, spanning from endodermal differentiation to induction of apoptosis. The cellular programme keeping a certain set of cells uncommitted is, however, not understood.

With the tools generated for this thesis, it should be possible to look at signalling activities of STAT3 and Smad1 in real time during early embryogenesis, but also during other processes involving stem cells, such as regeneration of injured tissue or cancer initiation. Together with the constantly improving imaging techniques and the easy accessibility of fish embryos, it should be possible to study the molecular control of pluripotency in less artificial setups.

A first, small step in that direction are the correlative analyses of signal transducer localisation and function applied in this study in both early embryos and cell cultures. Adding to the value is research data on human and monkey ES-cell cultures. By comparing results from research on four evolutionarily quite distant vertebrate species, it will may be

possible to filter out species-specific peculiarities and hence focus on the common basis of molecular pluripotency control.

2. Results

2.1 The role of STAT3 and BMP signalling in different stem cell systems

2.1.1 Identification and Cloning of STAT3 cDNA of the Medaka Oryzias latipes

Comparison between evolutionarily distant vertebrate species is a useful approach to evaluate the evolutionary pressure applied on a genetic system, such as a signalling pathway and its direct transcriptional targets.

For a comparative approach between Medaka and mouse it was crucial to be able to assess the status of signalling pathways known to play decisive roles in pluripotency control. Hence a first goal was to identify, clone and functionally compare the central transducer protein STAT3.

Using the human protein sequence of STAT3 (http://www.ensembl.org/Oryzias_latipes/index.html), as query for a blast search of the Ensemble Medaka genome database , the Medaka orthologue of human STAT3 was identified:

http://www.ensembl.org/Oryzias_latipes/geneview?gene=ENSORLG00000004061;db=core

The predicted sequence identity on protein level was 84% between human and Medaka. The amino-acid alignment shown in *figure 5* highlights long stretches of high sequence similarity and two stretches of significant difference.

Figure 5: Multiple STAT3 protein alignment of different species. The alignment is color-coded according to the aminoacid type.

Between AA120 and AA230, a series of single amino-acid exchanges between the mammalian STAT3 proteins and the Medaka sequence are obvious. Many of these changes are, however, to structurally conservative amino-acids and there are no insertions or deletions. Hence, it was assumed that the protein function is not significantly altered by these changes in the N-terminal region. In the C-terminus however, there is a stretch of strong difference between Medaka STAT3 and the mammalian counterparts. First, there is a 20AA insertion in the Medaka peptide around AA720. Then there is a series of non-homologous changes and gaps afterwards. Interesting is, however, that the major sites for protein modification known for mammalian STAT3 are conserved in this low similarity region. The tyrosine residue (mammalian tyrosine 705) is the prime target of activating phosphorylation, upon which dimerisation of STAT3 occurs and leads to nuclear accumulation and transcriptional activity. There is a 12AA long stretch of perfect homology surrounding this residue, directly followed by a 20AA insertion. The other site of

phosphorylation in the mammalian protein is Serine727. At this position, there is again a 10AA long stretch of perfectly matching residues between all analysed sequences. These findings suggest that the regulation on protein level is likely conserved at both these sites, and again raises the probability of functional conservation.

The genomic locus of STAT3 in mammals is quite interesting, as another gene of the STAT family, STAT5A, is located directly adjacent, but in reverse direction to STAT3 [57]. In zebrafish, more orthologues have been identified, with one showing a similar genomic organisation[58] (http://www.ensembl.org/Danio_rerio/multicontigview?s1=hs&seq_region_right=12970348&click_right=893&click_left=110&seq_region_strand=1&seq_region_name=3&h=ensdarg00000019392&seq_region_left=12469849&seq_region_width=43908&click.x=499&click.y=70).

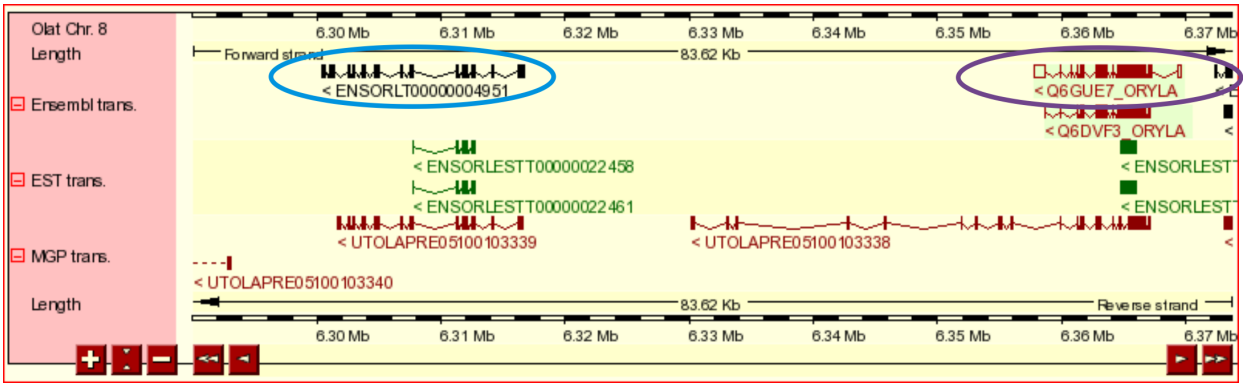

Figure 6: Graphical respresentation of the genomic region surrounding Medaka STAT3. Hightlighted by a purple circle on the right side of the scheme is the thus far not annotated Medaka orthologue of STAT3. Further to the left, about 50 kilobases away in inverse orientation the predicted orthologue of the mammalian STAT5A is marked by a blue circle.

When searching for the corresponding loci in the Medaka genome assembly, a very similar situation was found. Gene prediction identifies two Medaka orthologues of human STAT5A. One of them (http://www.ensembl.org/Oryzias_latipes/geneview?gene=ENSORLG00000003961), best matching the zebrafish stat5.1 gene, maps juxtaposed to the previously identified MFSTAT3 locus (*figure 6*, compiled using ensembl and available at this URL: http://www.ensembl.org/Oryzias_latipes/multicontigview?region=8&vc_start=6300025&vc_end=6373646&s1=dr&h=ensdarg00000022712%7Censorlg00000004061).

These findings represent additional leverage to the conservation of the gene. No second copy of STAT3 was found in the Medaka genome. The genomic organisation of the STAT3 loci of Medaka mouse and human are highly similar, their main feature being the close and

inverted positioning of STAT3 and STAT5. All these data on the genomic site and the protein conservation make a functional conservation of STAT3 highly likely.

Based on the bioinformatic analyses, MF STAT3 was cloned by PCR from Medaka cDNA prepared from a mixture of embryonic stages. Different sets of primers were used. One resulted in a stop-codon free product that was cloned either as a fusion with GFP or mOrange (mf_stat3-f04 with mf_stat3-r04), two spectrally different fluorescent proteins that have been proven to not inhibit mammalian STAT3 function when C-terminally fused [33]. Additionally, a FLAG-tag sequence added at the C-terminus right before the stop-codon was introduced (full sequences are available online in the LabWorld database at http://labworld.cyrap.de).

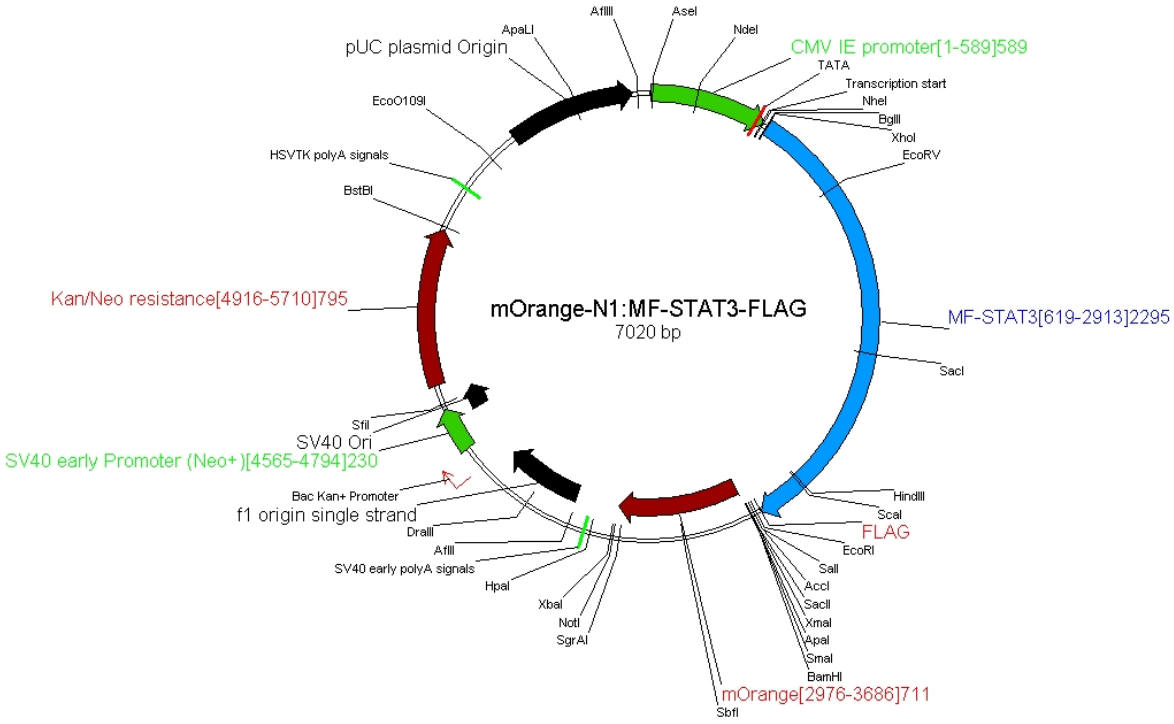

Figure 7: Plasmid Map of the MFSTAT3-mOrange fusion protein vector pmO-MFSTAT3. Using the free software VectorNTI, a plasmid map based on a complete sequence is depicted. Shown in light blue is the fusion protein consisting of the full length MFSTAT3 fused in frame to the short FLAG epitope-tag sequence and the bright monomeric fluorescent protein coding sequence of mOrange.

As the STAT3 antibody (SCBT, Stat3 C-20: sc-482) used for this study is polyclonal, targeting a sequence of mixed homology at the C-terminus of mammalian STAT3 (AA719-769), its binding fidelity was tested by ectopic overexpression of the cloned and GFP-fused MF-STAT3 in human 293T cells, followed by Western blot. The corresponding *figures 8* and

9 verify successful cloning and expression of the MFSTAT3-GFP fusion protein. The Western blot experiments detect bands of correct size after probing with STAT3, GFP and FLAG antibodies specifically in MFSTAT3-GFP transfected cells.

It should be noted that Medaka STAT3 can only be detected in 293T cell lysate by the STAT3 antibody used throughout this thesis after the endogenous STAT3 is cut off. This fact hints at a present, but weak affinity of the antibody against the Medaka protein. The FLAG and GFP antibodies used in the blots shown below detect the fusion protein indifferent of the endogenous STAT3 being present on the membrane.

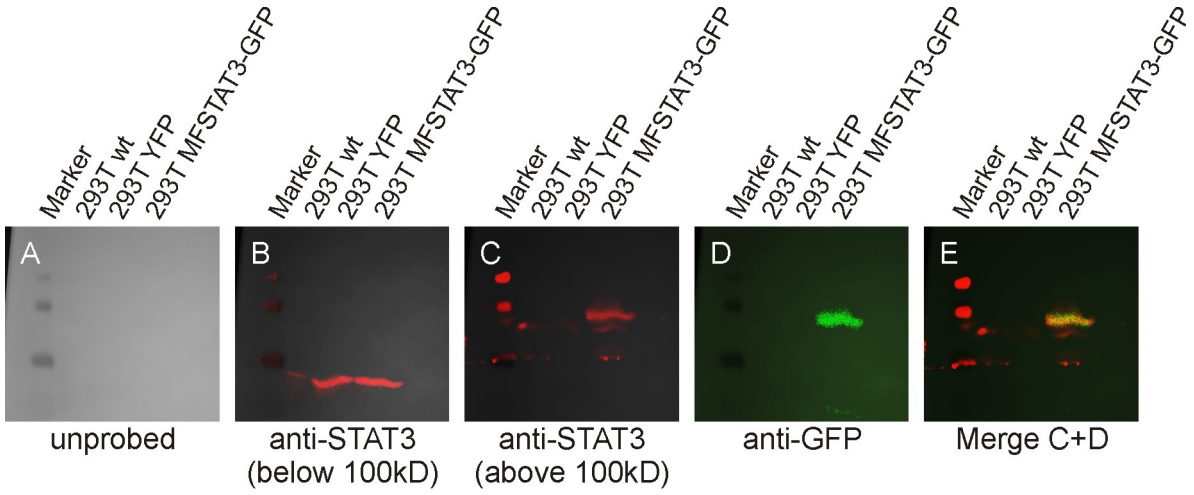

Figure 8: Detection of the MFSTAT3-GFP fusion protein in Western blots. (A) shows the unprobed membrane after Western blot. (B) represents the endogenous STAT3 detected by probing the complete membrane. (C) shows the MFSTAT3 detection with the STAT3 antibody is readily detectable in the lysate of cells previously transfected with the described MFSTAT3-GFP plasmid. In (D) the same band is visualised by probing the blot with an anti-GFP antibody, verifying the fusion. (E) shows the overlay of GFP and STAT3 detected bands.

Using recently established fluorescent Western Blot detection with a CCD-camera device (Kodak IM6000), it was possible to detect different epitopes of one protein or different proteins running at the speed and blotted to the same level of the membrane. In the presented case, the rabbit-derived anti-STAT3 primary IgG was detected by a secondary antibody coupled to the infrared emitting fluorochrome IRDye800 (*figure 8C*). At the same time, a mouse-derived anti-GFP primary antibody was concurrently detected at about 125kD by a secondary anti-mouse-IgG antibody coupled with the fluorochrome Alexa594 (*figure 8D*), resulting in the overlay of both channels (*figure 8E)* clearly demonstrating expression of the expected fusion protein. This result was further confirmed by probing an independently prepared Western blot membrane with an anti-FLAG antibody (figure 9) to detect the corresponding epitope that was introduced between MFSTAT3 and GFP during

cloning. As the CCD-camera captures signals with a 16bit intensity range per pixel, the sensitivity and signal strength differentiation of this method exceed standard methods by several multitudes. All images are pseudo-coloured to palettes for red and green to better visualize the overlapping bands.

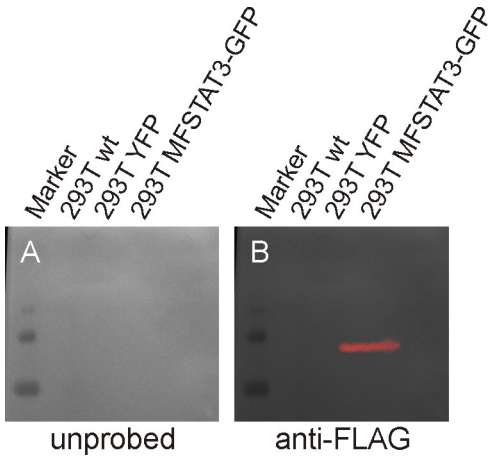

Figure 9: Western blot detection of the MFSTAT3-GFP fusion protein through its FLAG-epitope tag. In (A) the membrane resulting from a replica of the previous Western blot is shown. In (B) a band of the expected size of the MFSTAT3-GFP fusion protein is seen after FLAG antibody probing.

Using this fusion protein, analyses of the signal activity of STAT3 in stem cell cultures was possible in living cells. Hence, the availability of a stable embryonic stem cell line (Mes1) derived from the blastula stage of the japanese Medaka fish *Oryzias latipes* was used. A comparison of STAT3 localisation in Mes1 cells and in the embryonic stage corresponding to their derivation, the late blastula, was conducted first.

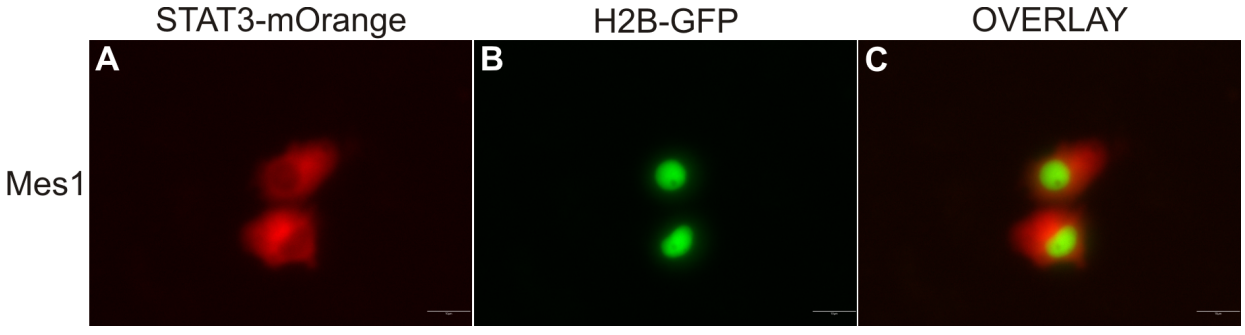

Figure 10: Live-imaging of Mes1 cells expressing H2BGFP and MFSTAT3-mOrange. Mes1 cells growing under standard conditions co-transfected with MFSTAT3-mOrange and H2BGFP are shown. In (A) only the mOrange signal is detected almost exclusively in the cytoplasm, while (B) marks the nuclei with GFP signal. In (C) the merge of (A) and (B) is given.

Mes1 cells were transfected with a mixture of two plasmids. One plasmid was used to drive expression of a fusion between the Histone2B and GFP. This fusion was obtained from Dr. R. Köster and is described as a label for the nucleus [59]. In order to be able to discriminate the STAT3 fluorescence from the nuclear Histone-fusion signal, MF-STAT3mO was used as the second plasmid. After 24 hours, the transfected Mes1 cells were analysed using an inverted fluorescence microscope. Typical examples are presented in *figure 10*. The GFP signal was exclusively localised to the nucleus, while the mOrange signal is exclusively cytoplasmic. The weak red fluorescence in the nuclei is due to the imperfect optical sectioning of a normal inverted microscope and probably originates from cytoplasmic areas above and below focal plane cutting through the nucleus.

Notably, the localisation of STAT3 changed to predominantly nuclear when the cells were seeded on cover slips either coated with collagen (*figure 11*) or gelatin (*figure 12*). Even more striking is the nuclear sublocalisation of STAT3 which strongly differs between Mes1 cells seeded on either collagen and gelatin coated plastic. While the gelatin coated surface leads to accumulation of STAT3 in low DNA-concentration areas (probably nuclear bodies, pers. comm. Dr. R. Hock), the collagen coating results in a rather diffuse distribution in the nucleus and also in the cytoplasm adjacent to the nuclei.

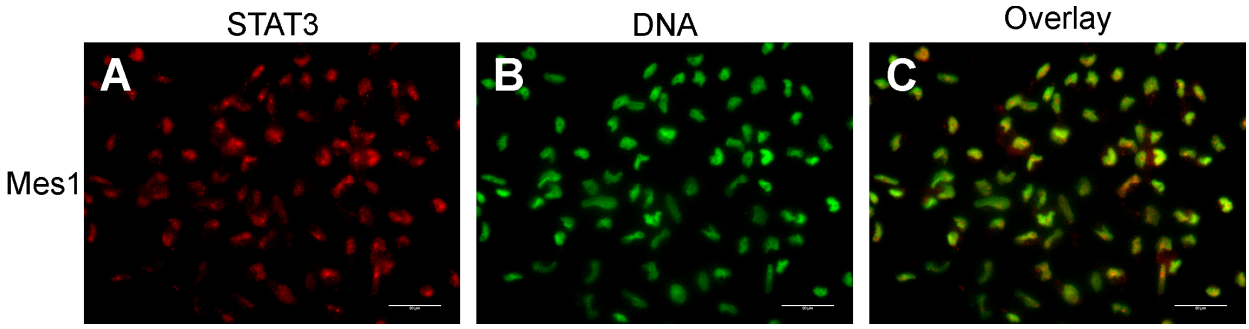

Figure 11: STAT3 localisation changes upon seeding on collagen-coated coverslips. Immunofluorescence against STAT3 (A) and DAPI staining for DNA (B) are strongly overlapping (C). The Mes1 cells used for this assay were grown on coverslips coated with collagen.

Generally, only a small fraction of cells attached to either of these surfaces. The described phenomenon may be explained either by a gained ability to attach to these surfaces as a result of nuclear STAT3 in a small subpopulation of Mes1 cells, or, the few cells able to attach did react to the surface change by activating STAT3 and translocating it to the nucleus.

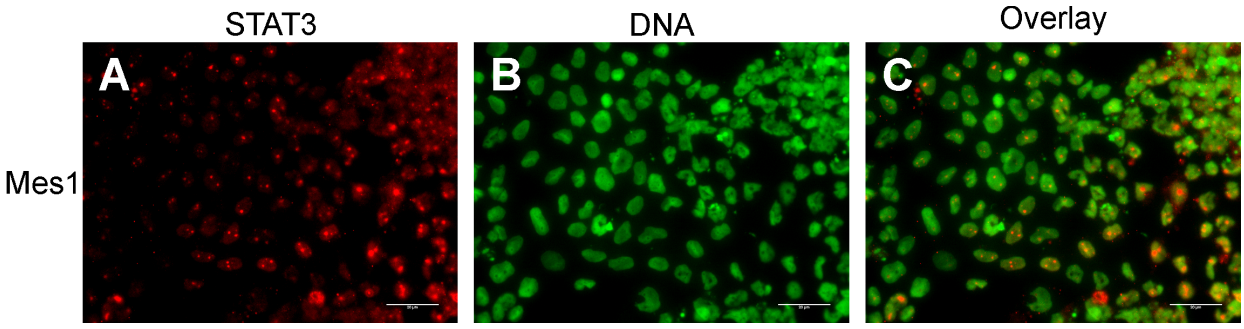

Figure 12: STAT3 localisation is affected by seeding of Mes1 cells on gelatin-coated coverslips. Shown are representative examples of immunofluorescence stainings for STAT3 in red (A) and DNA in green (B). (C) is a merge of (A) and (B).

2.1.2 Analysis of the activity of STAT3 during early embryogenesis

The *in vitro* signal status was compared to the *in vivo* situation by looking at the origins of the stem cell systems. The Mes1 cells were derived from high blastula Medaka embryos. First a series of embryonic cDNAs was synthesised and subjected to PCR for MF STAT3 expression (*figure 13*). RNA levels of STAT3 are rather stable throughout development. The PCR bands are a result of mature mRNA as evidenced by absence of a band when no template cDNA is present and a shift of the band when genomic DNA is used.

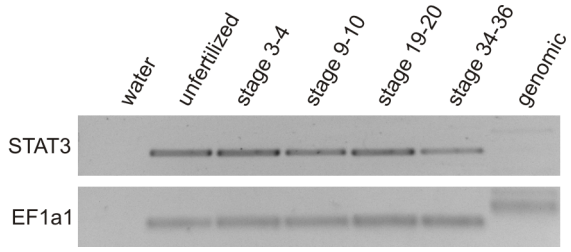

Figure 13: mRNA expression of MFSTAT3 during Medaka development. The upper row of the RT-PCR reaction on different stages of Medaka embryogenesis show STAT3 constantly present in all stages including the maternal phase. The lower row represents expression of the housekeeping gene EF1a1.

Importantly, even in unfertilised eggs STAT3 mRNA can be readily detected, suggesting maternal deposition of mRNA and early presence of protein. To verify protein presence and investigate signalling status during early development of Medaka, immunofluorescence for STAT3 on different stages was performed. Then, mRNA of the MFSTAT3-mO fusion protein was synthesised *in vitro* using the mMessage Machine kit (Ambion) and injected together with mRNA derived from the above described vector encoding the H2B-GFP fusion. In all

analysed stages, the immunofluorescence signal was perfectly corresponding to that generated by direct immunofluorescence.

STAT3 localisation in a Medaka blastula embryo is exemplified in *figure 14*. Analyses showed that STAT3 is exclusively cytoplasmic in all imaged cells. Hence, STAT3 is not active during the period of Mes1 cell culture derivation.

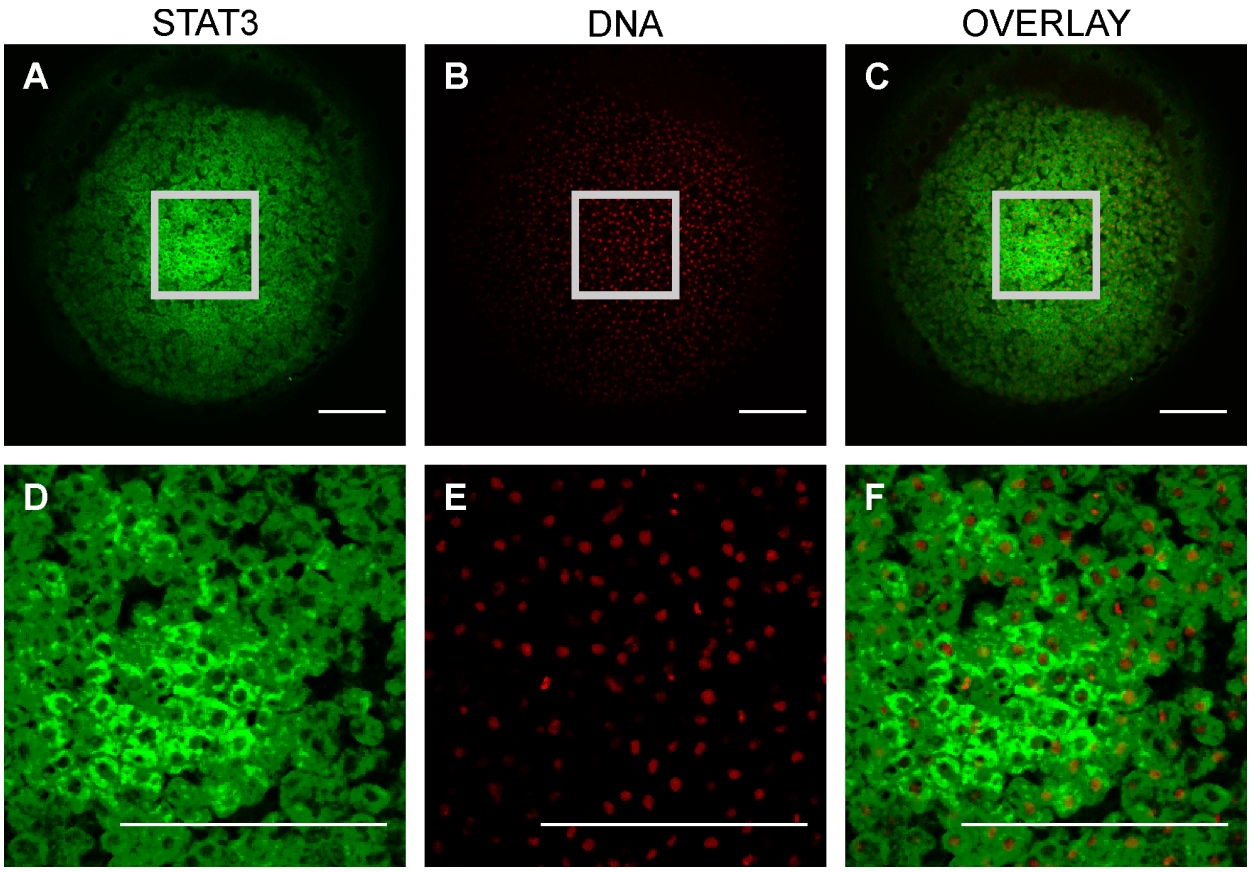

Figure 14: A representative optical section through a Medaka blastula embryo stained for STAT3 and imaged by confocal microscopy. In (A/D) The anti-STAT3 signal of a optical section through the center of a Medaka blastula is shown, pseudo-coloured in green. The nuclei of the cells are shown in (B/E), visualised by red colour. Graphical overlays of both signals are given in (C) and (F). Indicated by the white rectangle in pictures (A-C) are the areas magnified for a more detailed, digitally zoomed view (D-F) where the clear exclusion of STAT3 signal from the nuclear areas can be seen. All scale bars in this figure represent 250µm.

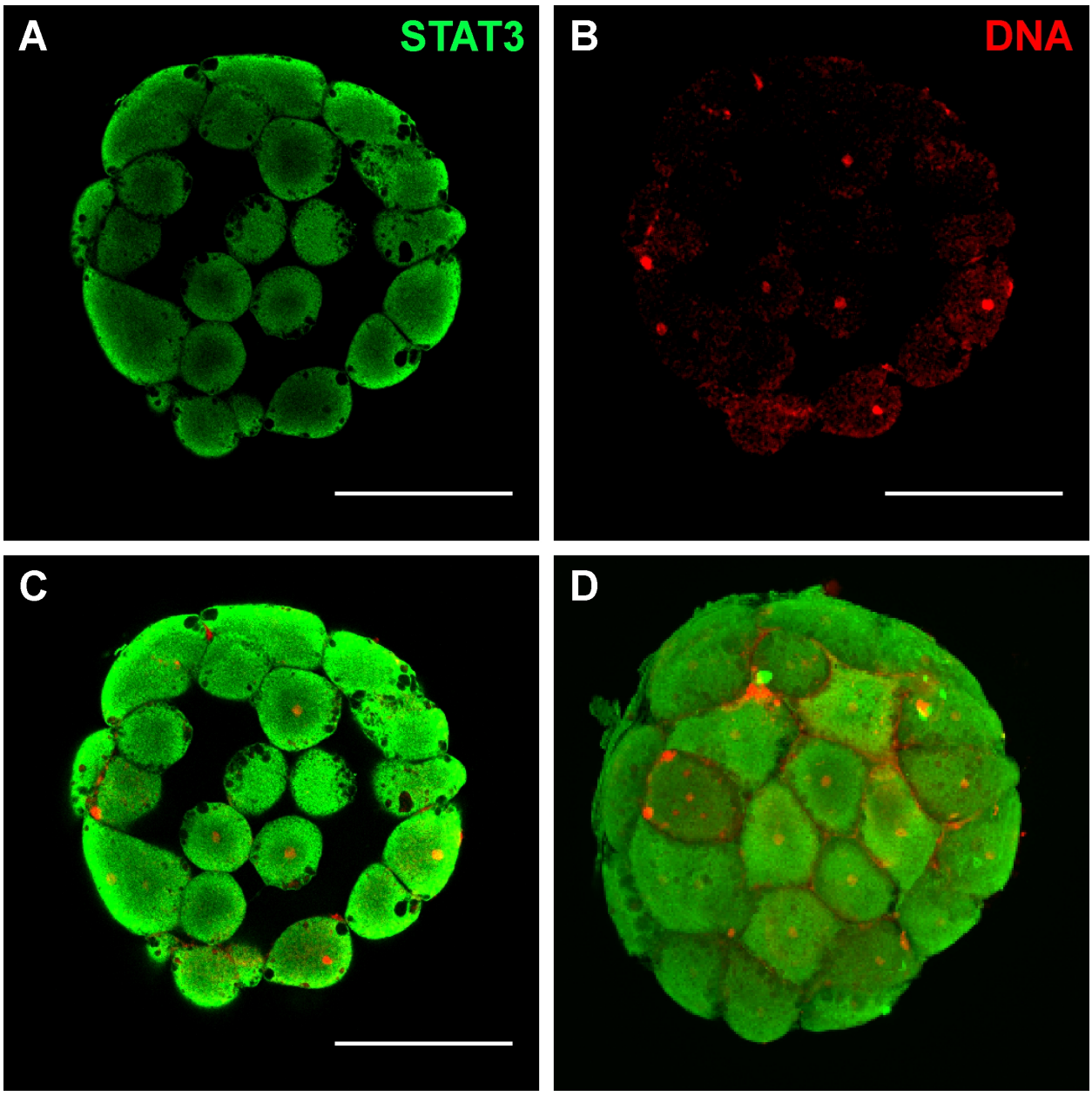

Figure 15: STAT3 is only cytoplasmic in a 26-cell Medaka embryo. An example confocal section through a 26-cell stage Medaka embryo stained for STAT3 (A) in green and DNA (B) in red. The overlay of both channels (C) and the 3D-projection (D) of all 256 confocal images per channel reveal broad cytoplasmic distribution of STAT3. Scale bars represent 250µm.

Next, earlier stages of development were analysed with the same approach. Interestingly, STAT3 was found to accumulate in the nuclei of cells during a period between 64 cell stage and 512 cell stage. Figure 16 represents an example of an embryo with a mixture of cells showing nuclear or cytoplasmic concentration of STAT3, while the 26 cell stage embryo shown in figure 15 is still devoid of nuclear STAT3. This finding was verified by a series of embryos with STAT3 visualised by either immunofluorescence or mOrange fusion.

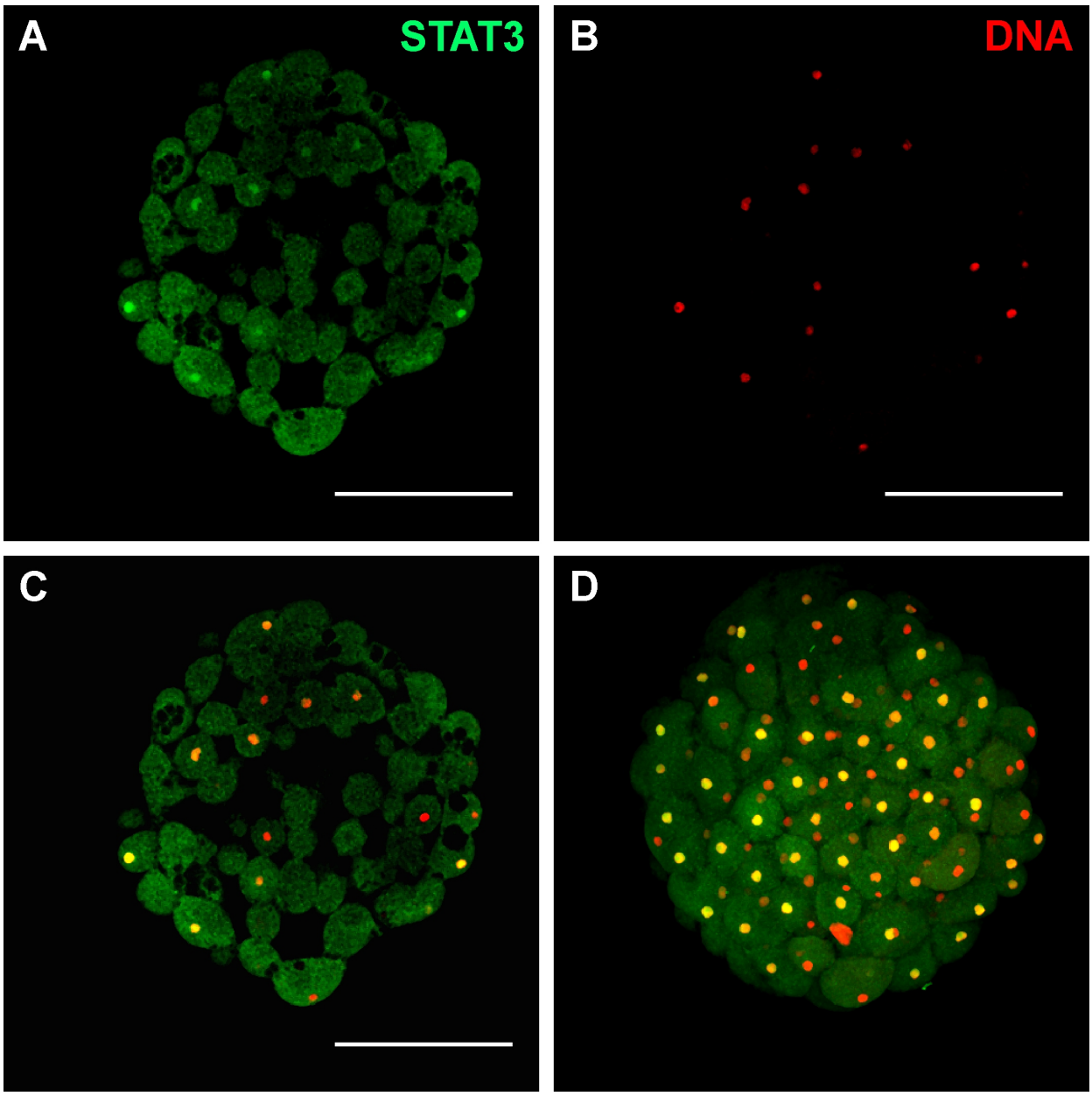

Figure 16: Example of a Medaka embryo at 114 cell stage with mosaic STAT3 activation. (A) shows a STAT3 immunofluorescence (green) experiment on a 114-cell stage embryo. In (B) The same confocal slice showing the DNA stain (red). (C) depicts the overlay of both signals. All 256 optical sections for both channels are projected in (D). All scale bars represent 250µm.

In summary, the signalling status of STAT3 in Medaka appears to be inactive in both embryo and derived cell culture system.

On the other hand, murine stem cell pluripotency is strongly dependent on nuclear, active STAT3 [48]. Mouse stem cells are usually derived from 3.5dpc embryos. Only the inner-cell-mass (ICM) cells are pluripotent and give rise to ES-cell lines. Therefore, embryos of this stage were used for immunofluorescence against STAT3. Using a confocal microscope, high-resultion images allowed exact determination of STAT3 localisation in 3D. As demonstrated by *figure* 17, STAT3 is localised in the nuclei of the ICM cells, and should hence be considered active.

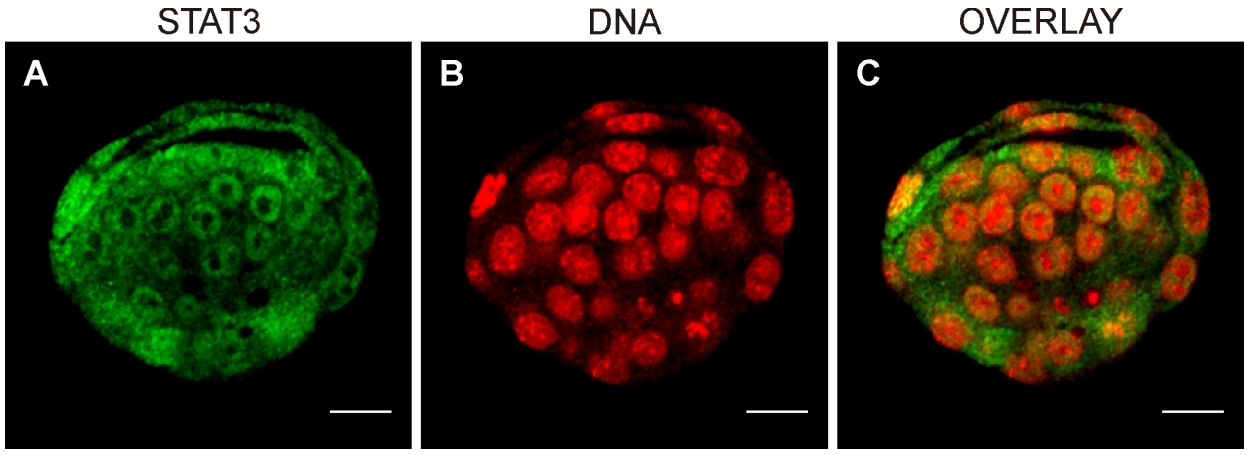

Figure 17: STAT3 activity in 3.5dpc mouse blastocysts. A mouse blastocyst embryo was stained by immunofluorescence for STAT3 in green (A) and for DNA in red (B). An overlay of both channelsof this virtual section is shown in (C). Scale bars represent 20µm.

It was not possible to derive enough embryos to compare a series of different stages as done before with Medaka. In any case, the presence of STAT3 in the nuclei of the ICM cells and the continued dependence of these cells on nuclear STAT3 when cultured represents a striking difference between the two model animals analysed here.

2.2 MAP-kinase signalling in Mes1 cells

As one of the most prominent signalling pathways known, the status of the MAP-kinase pathway was evaluated in different stem cell systems, including Mes1 cells. STAT3 was previously shown to be co-regulated by members of the MAPK pathway [60]. Western blots using phospho-specific antibodies to determine activation of different branches of the MAPK signalling pathway in different cell types, including murine embryonic stem cells (WW6) and Medaka embryonic stem cells (Mes1). Both, the p38 branch and the MEK1/2 kinases are linked to STAT3 [61][60][62][63], and p38 is also associated with a branch of BMP2 signalling [64][65][66].

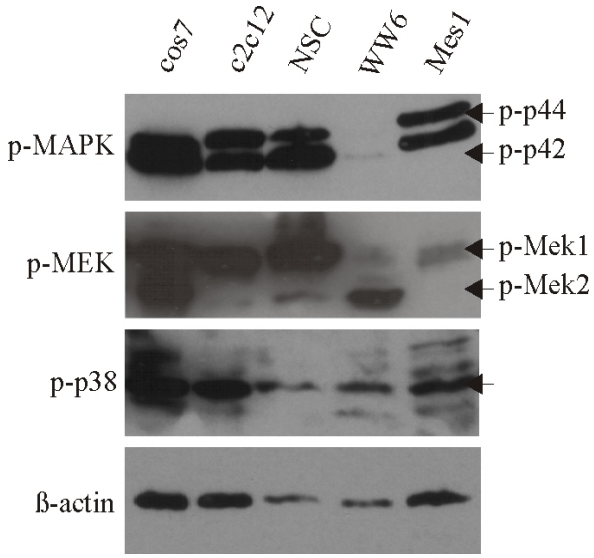

Figure 18: Western blot analyses of phosphorylation status of MAP-kinase pathway proteins. Phospho-specific antibodies were used on lysates of different cell lines as indicated above. Rows 1 to three were probed with different phosphorylation-specific antibodies against proteins of the MAP-kinase signalling pathway. Row 4 shows the loading control for protein amounts used. On the right side arrows indicate the protein-specific bands, as two MAPK variants and also two MEK variants are detected by the respective antibodies.

Interestingly, the so-called stress kinase p38 is active in all tested cell lines (*figure 18, row 3*). At the same time, MEK1 phosphorylation is weak in WW6 and Mes1 cells. p-MEK2 is either not bound by the antibody in Mes1 cells or not activated. Mouse ES-cells (WW6) are the only cell line tested where MEK2 phosphorylation strongly exceeds the p-MEK1 signal. It should be considered that antibody binding affinities might vary between mouse, human and Medaka proteins. However, the overall conservation of the MAPK pathway proteins is very high between these species. Antibodies against the activated forms of the MAP-kinases p42 and p44 - targets of MEK1/2 and cytoplasmic-nuclear transducers of the

pathway - clearly show a strong activation of the MAPK pathway in Mes1 cells, but low levels of activation in mouse embryonic stem cells (*figure 18, row 1*). The strong phosphorylation in Mes1 cells can readily be explained by the presence of bFGF in the standard culture medium. This is also present in the primary mouse neural stem cell medium, again reflected by strong p42/44 bands in the NSC lane of the Western blot. For the cell lines cos7 and c2c12 the reason for signal activity must be intrinsic as the medium used for their culture does not contain growth factors. The fact that WW6 cells show only very weak p-p42 levels, but considerably stronger p-MEK levels is puzzling. These results suggest that the very high cell-cycle rate of mouse stem cells could be largely independent of the MAPK pathway or that the transduction mechanism of this pathway is rerouted in these cells.

With respect to STAT3, these results implicate that a MAPK-pathway activation is not directly, positively connected to STAT3 activation in either mouse or Medaka embryonic stem cells. In contrast, STAT3 localisation is inverse to p42/p44 phosphorylation in both cell types, namely cytoplasmic in Mes1 cells and strictly nuclear in WW6 cells. Whether this observation is incidental or due to a negative crosstalk in embryonic stem cells could not be clarified to this end.

STAT3 tyrosine phosphorylation, and thereby nuclear accumulation, has been reported to be negatively affected by p38 activity. p38 activation in both Mes1 and WW6 cells does not allow for a conclusion on crosstalk effects with STAT3 without further functional studies.

2.3 Determination of BMP cascade status in Medaka stem cell systems

BMP2-Smad1 signalling together with LIF-STAT3 signalling was described by Ying et al. [52] to be sufficient to maintain the stem cell status of mouse embryonic stem cell cultures. As described above, STAT3 is not nuclear in Mes1 cells. This cytoplasmic localisation directly reflects inactivity of the signalling pathway. Other reports suggest that BMP activity without a compensatory LIF-STAT3 signal will lead to differentiation.

Attempts to localise Smad1 with an antibody from Santa Cruz Biotechnology did not give any staining in either immunofluorescence or Western blot experiments (data not shown). Hence, a GFP-tagged variants of human Smad4 (the construct was a gift from Dr. CS Hill, [67]) was transfected into Mes1 cells. As with STAT3, Smad4 localisation was thus trackable in living cells, and again the majority of protein was cytoplasmic (*figure 19*), suggesting inactivity of the BMP-Smad pathway.

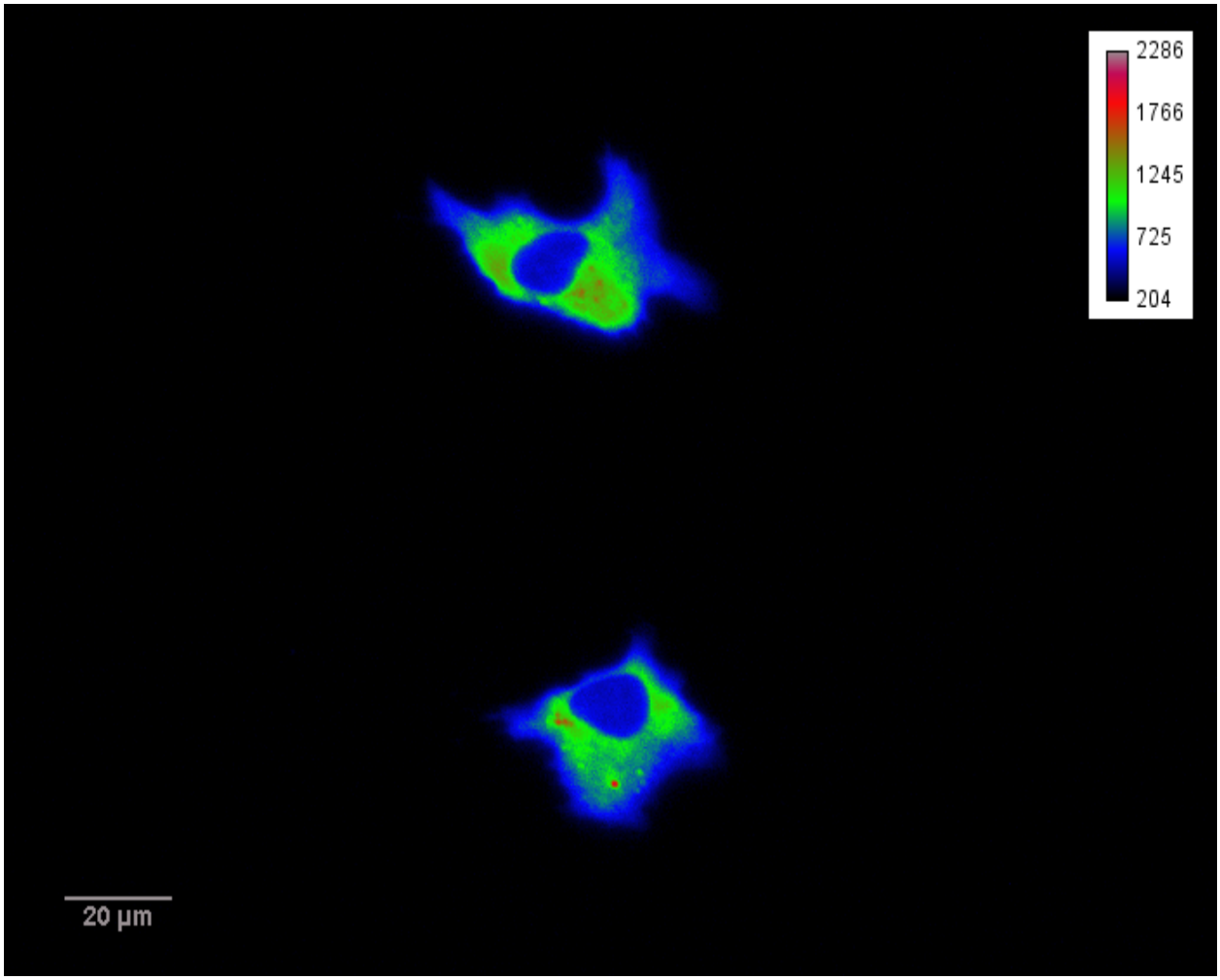

Figure 19: Human Smad4-GFP fusion protein is cytoplasmic in Mes1 cells. Live Mes1 cells under standard medium conditions are shown with a color-coded intensity scheme (RGB Rainbow LUT indicated in the top-right corner).

A step further, activation of immediate target genes of BMP signalling was analysed. For that, the inhibitor of DNA binding 2 (id2) gene from Medaka was cloned and analysed by RealTime PCR before and after stimulation of Mes1 cells with BMP2 and an inactive form of BMP2 called A34D. This BMP2 variant was shown by Nickel et al. [68] to have no binding activity to BMP-receptor II, while it retains full affinity to BMP receptor Ia. Furthermore, it was reported to be inhibitory for Smad signalling.

Standard PCR on cDNA from Mes1 cells revealed readily detectable expression of *id2* under normal culture conditions. A triplicate of real-time PCRs for untreated, BMP2 treated and BMP2-A34D treated Mes1 cells harvested two hours after ligand addition are presented in *figure* 20: Relative to the basal transcript level, the addition of BMP2 induces a more than four-fold increase within two hours. BMP2-A34D treated cells did not show altered

expression levels of *id2* compared to the level of untreated cells (same delta Ct relative to *ef1a1*).

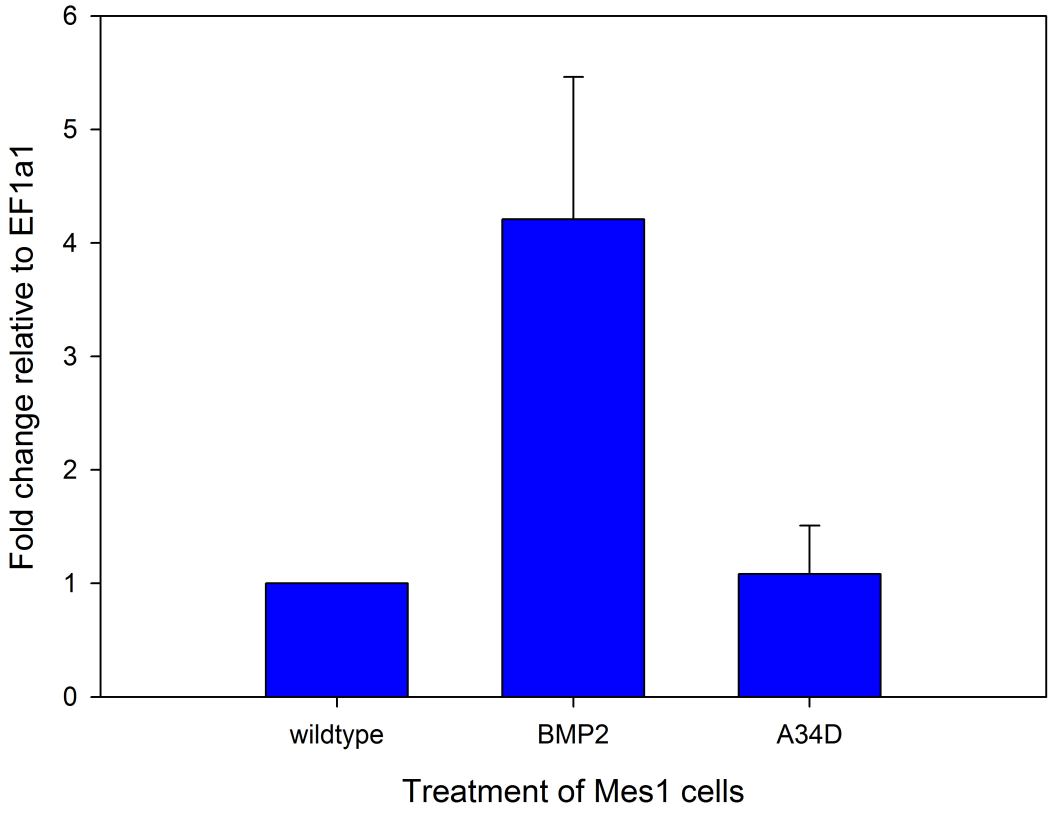

Figure 20: Quantitative Real-Time PCR for id2 on differently treated Mes1 cells. The graph shows averages of triplicate qRT-PCRs for id2 relative to the expression levels of the housekeeping gene EF1a1.

These data suggest a basal BMP-signal independent *id2* expression in Mes1 cells. While Smad4-GFP is cytoplasmic in untreated Mes1 cells, *id2* expression is already detected, but can be boosted fourfold by additional BMP2 presence. Blocking of Smad signalling by the inhibitory BMP2-A34D is not able to lower basal expression levels. While binding of hBMP2-A34D to the Medaka BR1a receptor can not be proven due to technical reasons, the effect of hBMP2 strongly indicates successful binding. The sole mutation at AA34 of the ligand was shown to only affect affinities for BRII in the human system, and hence binding of the mutant variant in a transduction blocking manner is suggested. The use of competition experiments were not considered practical, as hBMP2 only binds its receptors in dimer-form and is consequently inhibited by the presence of both BR1a binding and non-binding dimer-

partners.

Smad4 was shown to play an important role in the nucleus during transcriptional regulation [69] of BMP signalling, but in Mes1 cells it appeared to be completely cytoplasmic. At the same time, a direct BMP signalling target gene, *id2,* which is involved in differentiation control, is expressed in the same cells. To clarify whether this situation was due to BMP-independent *id2* activation or due to problems with the human Smad4 protein used, further experiments were necessary.

Consequently, the Medaka orthologue of *smad1* was identified by Blast search. The reason for choosing Smad1 instead of Smad4 was its specificity for the BMP2/4 ligand induced signalling branch. Again, the protein conservation between human, mouse and Medaka was calculated after preparation of a multi-protein alignment (*figure 21).* With 89% sequence identity, and the presence of only one *smad1* gene in the Medaka genome, functional conservation is likely.

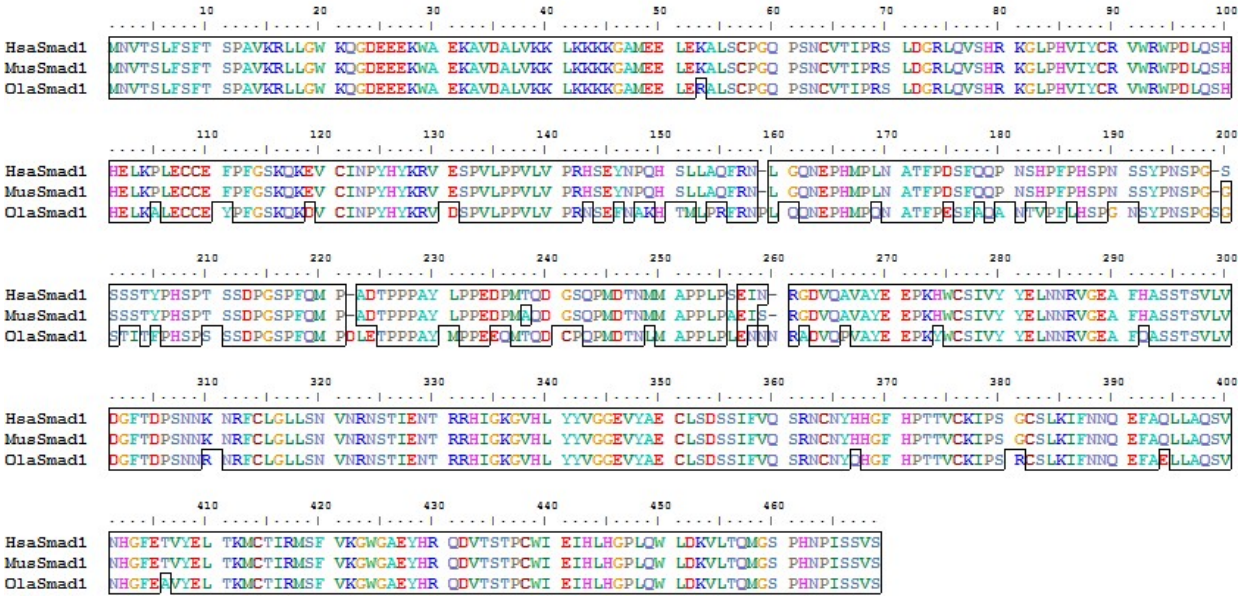

Figure 21: Multiple protein alignment of Smad1 of different species. The color-coded protein alignment compiled with ClustalW and BioEdit shows very high levels of sequence conservation between the two mammlian variants (human in line 1 and mouse in line 2) and the Medaka orthologue (line 3).

Consequently, the predicted coding sequence of MFSmad1 was amplified by PCR on cDNA prepared from mixed stages of Medaka embryos and cloned into pEGFP-C1 for use as a fluorescently tagged fusion protein in live imaging assays (*figure 22*). The full cDNA and

vector sequences are available online (http://labworld.cyrap.de).

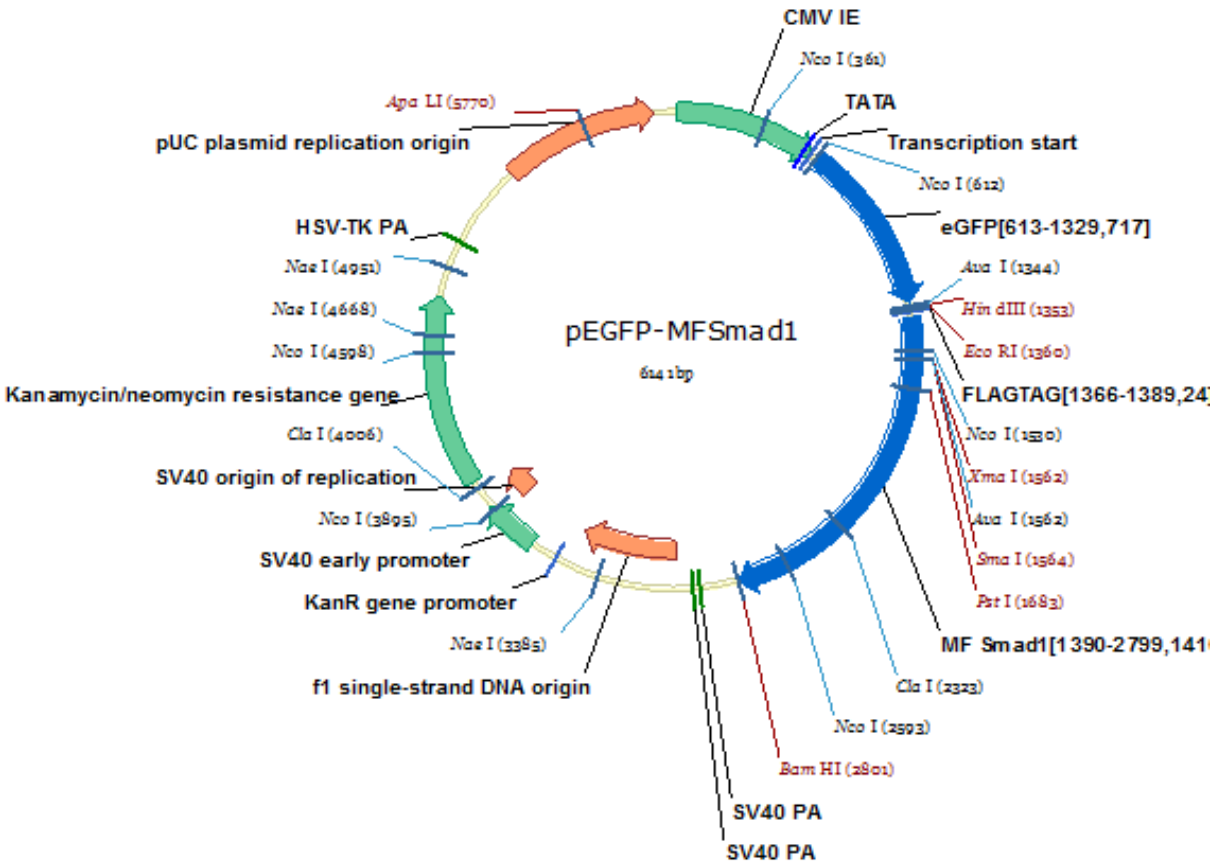

Figure 22: Plasmid map of MFSmad1 cloned as a fusion protein with a FLAG-epitope and eGFP. As reported in the literature, eGFP was fused to the N-terminus of MFSmad1. The complete fusion insert is shown in light blue.

Using this plasmid, Mes1 cells were again transfected and imaged live under standard culture conditions (*figure 23*).

Unexpectedly, the GFP-MFSmad1 fusion protein does predominantly localise to the nucleus under normal culture conditions. This finding contradicts the earlier described Smad4 exclusion from the nucleus, but fits the real-time PCR data on *id2*. It was not possible to clarify this situation further, but several explanations are feasible: It could well be that Smad4 is only needed for transporting Smad1 into the nucleus of Mes1 cells, but is then released from the complex and subsequently exported. Equally plausible is the scenario that human Smad4 - even though highly similar to its Medaka orthologue - is not correctly regulated when expressed in Mes1 cells, and therefore does not represent the status of the endogenous MFSmad4. Finally, it is possible that protein amounts of Smad4 are already

very high in Mes1 cells, and consequently extra levels induced by ectopic overexpression of the tagged variant accumulate in the cytoplasm even though signalling is active, but only to a degree able to translocate a minor amount of Smad4, but larger quantities of Smad1.

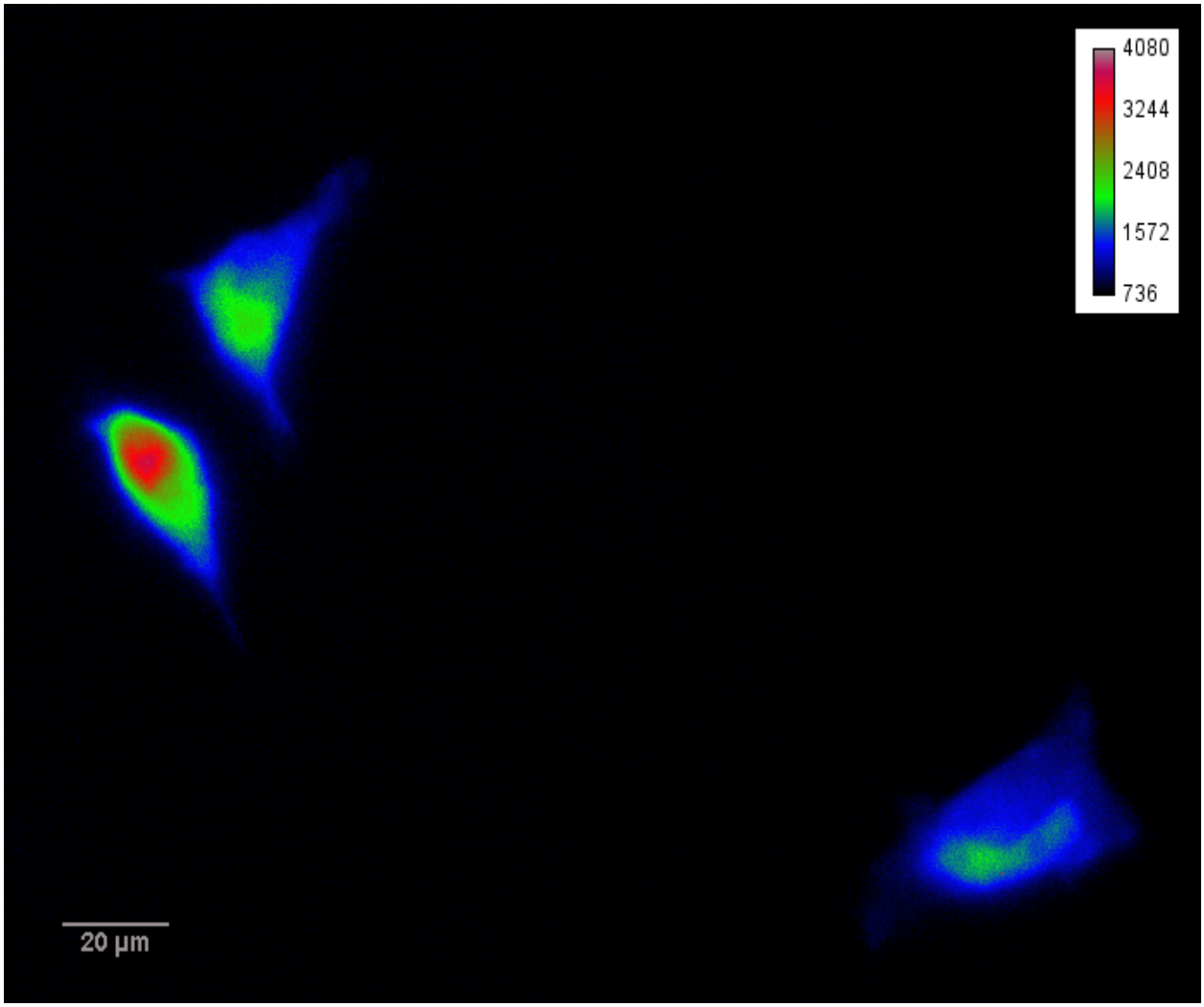

Figure 23: eGFP-MFSmad1 is predominantly nuclear in standard-culture Mes1 cells. Live Mes1 cells were transfected with eGFP-MFSmad1 and imaged in a standard cell culture dish. The picture is a typical example showing three cells with strong accumulation of eGFP-MFSmad1 signal in the nuclei. The intensity-coded color-scheme is annotated in the top-right corner (Rainbow RGB LUT).

2.4 Analyses of the interaction between STAT3 and BR1a

As mentioned before, several reports on the interaction of STAT3 and BMP signalling pathways in different stem cell systems have been published, the most prominent one dealing with the counter-regulation of differentiation programmes by negative transcriptional regulators [52]. This interplay between the two pathways is only very indirect via target gene regulation. In a different publication, a complex including Smad1, p300 and STAT3 was proven to be responsible for determining differentiation of neural stem cells [12]. Next to this instance of nuclear intersection of pathways in the form of competition for transcriptional co-factors, there is no hint at direct interaction of both pathways in the literature. Just like STAT3, BMP has a plethora of effects on different stem cells [70]. However, a detailed biochemical analysis of the molecular interactors of a possible cytoplasmic crosstalk between BMP- and STAT3 signalling pathways has not been conducted.

As a fist step towards this analysis, co-immunoprecipitation experiments of different components of the BMP signalling cascade and STAT3 were performed with protein lysates of differently treated mouse stem cells.

It was possible to detect complexation of STAT3 and BMP receptors by STAT3 precipitation with a specific antibody and subsequent probing with BMP receptor antibodies as presented in *figure 24*. These experiments were repeated several times with different materials (precipitation beads, antibody lots, lysates) always giving the same results.

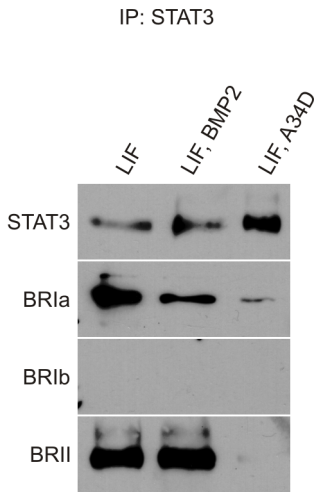

Figure 24: BMP-receptors are detectable in Western blots after immunoprecipitation of STAT3. IP of STAT3 was performed on ww6 mouse stem cell lysates after different treatments as indicated above the columns. Subsequent Western blots for three types of BMP receptors are shown in rows 2 to 4, while row 1 shows the precipitation and loading control.

Interestingly, the interactions between the different BMP receptors and STAT3 are destabilized when hBMP2-A34D is added to the medium. This mutant variant of hBMP2 was first described by Nickel et al. [68] to be a BMP2 antagonist as it shows no affinity to BMP-receptor II, but full affinity to BMP-receptor Ia. This results in blocked receptor heterodimerization and consequently inhibits Smad activation. The STAT3-pulled-down complex showed strongly reduced levels of BMP-receptor Ia, but no BMP-receptor II at all. Hence, the interaction between the BMP-receptor complex and STAT3 can be assumed to be driven by BMP receptor Ia or proteins bound to it.

In order to find out whether or not STAT3-BMPRIa complexation was an exclusive feature of embryonic stem cells, co-immunoprecipitation assays with cell-lysates from various other culture systems were done. As demonstrated by *figure* 25, it was possible to detect the complex in all tested cell types and even in Medaka embryos. It should be noted that all the experiments were performed with native cells-lysates, no ectopic overexpression of either STAT3 or BMP receptor 1a was necessary, indicating how abundant this complex must be.

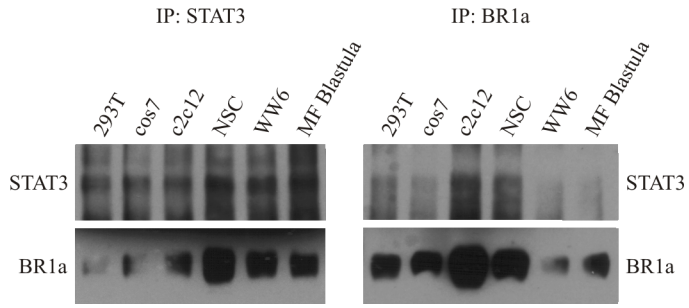

Figure 25: STAT3 and BR1a are co-precipitatable from diverse vertebrate cells. Shown are Western blots after precipiation of both, STAT3 (left side) and BR1a (right side), followed by probing with both antibodies.

The widespread detectability and high abundance of a complex made up of proteins involved in thus far isolated signalling pathways strongly suggests functional consequences of the complexation.

Binding of STAT3 to gp130 is dependent on a short peptide (YxxQ) motif identified by Abe et al. [21]. Analysis of the protein sequence of BMPR1a revealed two potential binding sites for STAT3 in the intracellular domain of the receptor. The first is located within the predicted Serine-Threonine Kinase domain at AA350 and matches the predicted binding pattern for STAT3:

Tyr-Gly-Thr-Gln (Y-x-x-Q)

The second site is inverted and closer to the C-terminus of BR1a: AA454:

Gln-Leu-Pro-Tyr (Q-x-x-Y)

Due to time limitations, it was not possible to verify specific binding of STAT3 to either site, but mutational analyses followed by co-immunoprecipitations should make it possible in the future to exactly determine the binding interface between both proteins if these sites are involved.

2.5 Functional consequences of the interaction between BR1a and STAT3

In order to assess the consequences of the interaction between BR1a and STAT3, mouse embryonic stem cells were starved (by deprivation of LIF) for 16 hours. Then, different combinations of growth factors including BMP2, BMP2-A34D, LIF and Noggin were added to the medium for one hour. STAT3 immunofluorescence followed in order to visualise STAT3 activity. Representative sample pictures of this assay are shown in *figure 26*.

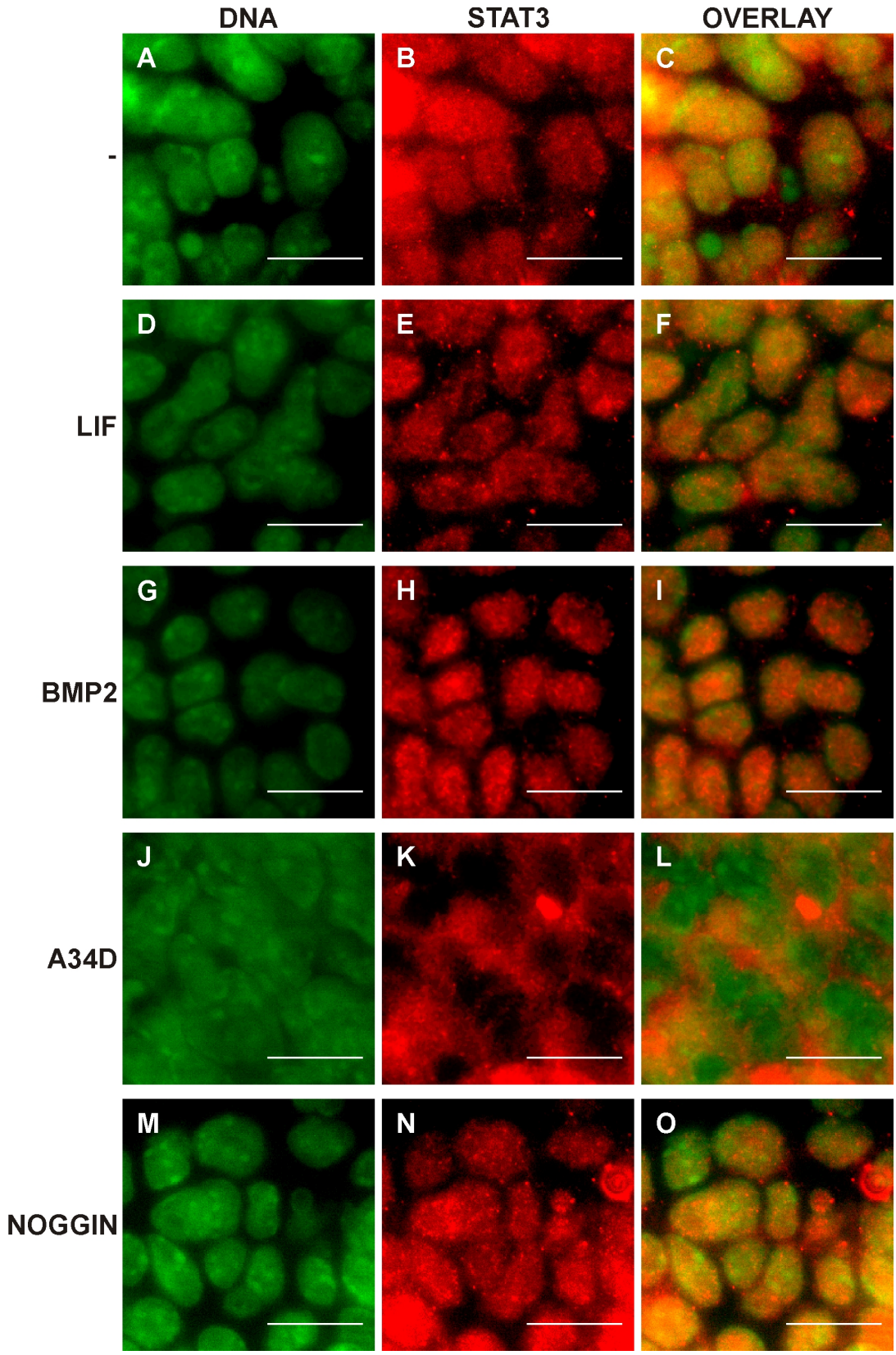

Figure 26: Treatment of LIF-deprived ww6 mouse ES-cells with hBMP2-A34D leads to cytoplasmic accumulation of STAT3. Images are typical examples of immunofluorescence assays stained for DNA (column 1, green) and STAT3 (column 2, red) after different treatments (indicated on the left) of LIF-starved ww6 cells. Scale bars represent 10µm.

Mouse stem cells usually grow in very dense clusters, are very small and have a very large nucleus and only little cytoplasm. These cells were grown feeder-free on 0.1% gelatin coated glass coverslips. They all originated from the same mother-well and were seeded at the same density. In all treatments except for the hBMP2-A34D row the strong majority of STAT3 signal (red) overlaps with the nuclear DNA stain (green). Upon blockage of BMP receptor heterodimerisation by hBMP2-A34D however, most of the STAT3 recedes to the small cytoplasmic ring around the nuclei (*figure 26 J-L*). As these images were taken on a normal inverted microscope, the optical sectioning is imperfect and light from non-focus depths is collected on the camera chip, leading to weak signals seemingly coming from within the nuclei, but actually originating from cytoplasmic regions above and below the focal plane. Also, the differences in growth and attachment between the cells of one image leads to some cells where the focal plane sections through the nuclei, while other cells are seen in cytoplasmic areas.

It is well established that Tyr705-phohsphorylation controls the cellular distribution of STAT3. Thus the same treatments as before were performed, but then the state of STAT3 was evaluated by immunoprecipitation, followed by Western blot specific for phosphoTyr705-STAT3 (*figure 27*). As expected, the long LIF-starvation leads to a strong reduction in STAT3 phosphorylation. Surprisingly however, phosphorylation is seen upon addition of BMP-receptor-1a ligands (BMP2 or BMP2-A34D). Not fitting the conventional view on STAT3 activity-control is the fact that phosphoTyr705 is strong when BMP2-A34D is added, but at the same time the localisation of STAT3 is predominantly cytoplasmic as demonstrated above.

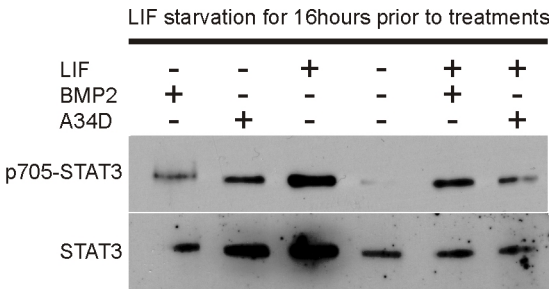

Figure 27: BMP ligand addition to LIF-deprived ww6 mouse ES-cells leads to phosphorylation STAT3. Immunoprecipitation was performed using a polyclonal STAT3 antibody followed by Western blot with an antibody specific for phosphorylated tyrosine705 of STAT3 (upper row) and controlled by back-probing with the precipitation-antibody (lower row).

As the BMP-receptor complexes have ony serine/threonine-kinase activities, while the antibody used for p-STAT3 detection is specific for Tyrosine705 phosphorylation it was assumed that other, so far unknown factors of the protein complex are responsible for this phosphorylation reaction. Furthermore, the subcellular distribution of STAT3 does not seem to be strictly dependent on the Tyr705 phosphorylation.

Both types of unexpected effects of BRIa-ligand interaction on STAT3, namely the Tyrosine705 phsophylation and the cytoplasmic accumulation upon A34D treatment, are unlikely to be directly mediated by the receptor itself, and no other known proteins were predcted to be good candidates for explaining the documented results.

Thus, an analysis of the protein complex containing both, STAT3 and BR1a was initiated. An estimation of the size of the complex containing both BR1a and STAT3 was done by High Pressure Liquid Chomatography (HPLC). The used column was calibrated with purified proteins of known molecular weight. The resulting elution volumes are shown as black squares and connected by a red line (*figure 28)*. Then, the sample experiment for STAT3-BR1a complex size estimation was run with 1mg of total protein of mouse stem cell lysate. The black triangles connected by the grey line in *figure 28* depict the amount of protein eluted with each fraction (1ml) as measured by the light-absorption at 280nm.

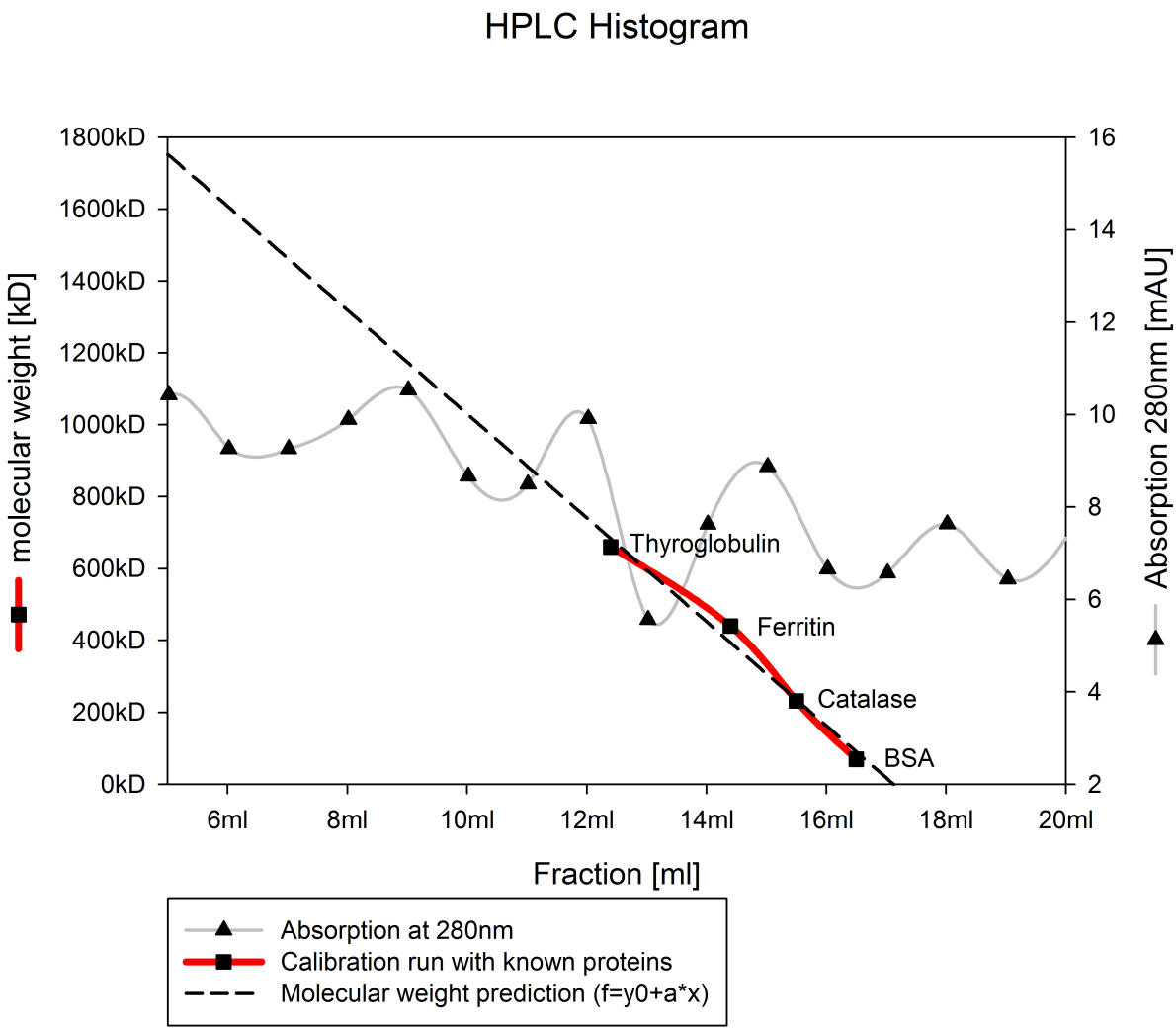

Figure 28: HPLC histogram of ww6 mouse stem-cell lysates and calibration with standard purified proteins. The grey line shows the absorption at 280nm of the fractions eluted after HPLC of ww6 lysate. The black block-symbols connected by red lines show the calibration runs with purified proteins of known molecular weight. The dashed line is the curve-fit prediction for estimating molecular weights of proteins and complexes eluted in earlier fractions.

The resulting fractions were then subjected to SDS-page and probed with antibodies against STAT3 and BR1a (*figure 29*).

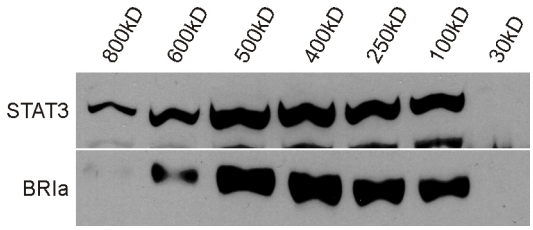

Figure 29: STAT3 and BR1a co-elute in fractions corresponding to molecular weights of 800kD down to 100kD. Western blots of selected HPLC fractions of ww6 lysates were probed with either STAT3 (top row) or BR1a (lower row).

The broad range of fractions with detectable amounts of STAT3 spans from the monomeric protein weight of around 90kD (fraction 16-17ml) up to an estimated 735-875kD (fraction 11-12ml). BMP receptor 1a is only weakly detectable in the 800kD complex range, but otherwise corresponds nicely to the fractions found positive for STAT3. As a negative control, the latest fraction collected, 23-24ml, was used which corresponds to proteins smaller than 30kD, the minimum separation size of the HPLC column.

3. Discussion

3.1 Medaka as an alternative model system for pluripotency control

In this study, the relevance of STAT3 and the BMP-Smad pathway in stem cell pluripotency has been determined. The mouse system is the by far best studied experimental vertebrate system available today. It also remains the only system where targeted genome alterations through homologous-recombination in ES-cell is possible. Furthermore, only mouse ES-cells have been experimentally proven to be germ-line competent – all other stem cell culture systems have either failed to contribute to offspring after re-implantation (Medaka [71], chick [72], rat [73], rabbit [74], dog [75], swine [76], monkey [77]) or have not been tested to this degree due to ethical constraints (human).

While STAT3 activity has been considered pivotal for mouse ES-cell pluripotency in culture [47], recent reports from laboratories working on monkey ES-cells and on human ES-cells clearly demonstrate that STAT3 is not needed to keep these cultures pluripotent [78][49]. With the here presented data on Mes1 cells and Medaka blastula embryos suggesting STAT3 inactivity, further leverage is added to these reports. However, mouse stem cells are still the by far best studied and technologically most advanced pluripotency system, and their seemingly isolated dependence on STAT3 signalling might very well play a role in their true and proven pluripotency which can not be attributed to the other systems.

At the same time, other molecular markers for stem cells such as Nanog [53][79] and Oct4 [80][81] are needed for stemness control in all thus far studied mammalian systems. As the only stable stem cell culture system of lower vertebrates, the Mes1 cells derived from Medaka blastulae represents a valuable tool for comparison of stem cell features. Considering the importance of controlling differentiation potential at all stages of development of multicellular organisms, it is commonly believed that the underlying molecular mechanisms have changed dramatically since fish split from the other vertebrates during evolution. The consequences of failing to maintain stem cells during development would be just as fatal as keeping cells in a dividing, pluripotent state for too long. Hence, I argue that differences between the stem cell culture systems are probably due to the artificial conditions used for maintaining the cells in the laboratory, but do not reflect changes to the basic, endogenous mechanisms guiding pluripotency *in vivo*.

When the expression of molecular markers for pluripotency in mammalian ES-cells were compared with that in Mes1, first fundamental differences were found: no *nanog* gene can be found in fish genomes [82] and the *oct4*-orthologous fish gene, *pou5f1*, is not expressed

in Mes1 cells, and the zebrafish mutant of the orthologous gene (“spiel ohne grenzen”, [83][84]) does not show similarity in phenotype to mouse oct4 loss-of-function.

Therefore, the experimental approach followed in this study, was the identification of active signalling pathways in the Medaka stem cell culture system.

3.2 The roles of STAT3 and BMP signalling cascades in stem cells

In most cases, signal transducers are latent transcription factors that are restrained by different means to the cytoplasm, where they are not able to influence transcription of target genes. Activation of a signal pathway then lifts the localising restrains of the corresponding transducer proteins and they accumulate in the nucleus, where they most often bind to specific DNA sequences and influence transcription – most of the time in an activating manner.

This system is used for the two pathways studied in more detail in this thesis. The signal transducer and activator of transcription 3 (STAT3) is such a latent transcription factor. Its localisation is indirectly regulated by a secondary modification, phosphorylation of Tyrosine705. This phosphorylation is induced by a wide variety of tyrosine-kinase enzymes, usually associated with cytokine receptors. Once phosphorylated, STAT3 monomers can homodimerise and thus refold in such a way that nuclear-import is enhanced while nuclear export is slowed down [36]. Thus, the phosphorylation finally leads to a nuclear accumulation. In parallel, the phosphorylation-induced dimerisation creates a DNA-binding interface, a clamp-like shape as evidenced by crystal-studies[28]. The direct target genes of STAT3 in embryonic stem cells have been studied [54] by microarray assays. However, none of the positively regulated genes appears to be directly associated with positive effects on pluripotency control. It rather seems like many of these target genes are actively repressing differentiation towards mesodermal and endodermal fates [52].

It is noteworthy, that several groups have proven STAT3-independence of human [50] and monkey [49] embryonic stem cell cultures. However, no studies on the corresponding embryonic stages have been published for these species.

Studies on BMP signalling in stem cells also hint at indirect effects on upkeep of pluripotency. Mechanistically similar to the STAT3 transduction cascade, the major pathway induced by BMP ligands makes use of latent trancription factors, the Smads. In their monomeric form, Smad1, Smad5 and Smad7 are not able to enter the nucleus. They are able to attach to BMP receptor complexes. Upon receptor activation by ligands, Smads

become phosphorylated and consequently associate with Smad4, the so-called co-Smad. The thus formed Smad-complexes are then actively imported into the nucleus, where they function as transcriptional activators. In detail, the regulation of Smads is far more complex, but has been elucidated in quite high detail [10][85]. However, the for this study most important feature of Smads is that their subcellular localisation is directly linked to their state of activity: cytoplasmic means inactive, nuclear corresponds to active. The same is true for the aforementioned STAT3. The BMP-Smad pathway is also not directly associated with positive effects on pluripotency. On the contrary, application of BMP to embryonic stem cell cultures readily leads to differentiation into mesodermal and endodermal fates. Quite fascinatingly, BMP does influence all kinds of stem cells *in vivo* and *in vitro*, with astonishingly diverse consequences. In the case of embryonic stem cells however, their main contribution to the maintenance of the undifferentiated state seems to be the activation of *id* genes [70]. These in consequence are then responsible for inhibiting DNA binding of a large set of differentiation inducing transcription factors including neurogenic factors such as Ngn1 and NeuroD [86][87].

The interplay of both signalling cascades in mouse embryonic stem cell cultures was studied by Ying et al. [52]. This publication further substantiated the idea of counter-inhibition of different differentiation pathways. While BMP signalling blocks neural differentiation through driving id expression, STAT3 target activation cancels out mesodermal differentation induced by other BMP target genes (*figure 4*).

3.3 Signalling activities in Mes1 cells

Whether this mode of cross-inhibition to maintaining pluripotency is corresponding to the *in vivo* situation is still unknown. Hence, the basis of this study was to characterise the activity-status of both pathways by immunofluorescence and also ectopic expression of fluorescent-protein tagged variants of the respective signal transducers, STAT3 and Smad1/4, in stem cell cultures derived from Medaka blastula embryos.

Both, GFP-hSmad4 and STAT3-GFP are almost exclusively cytoplasmic in standard-culture Mes1 cells. STAT3 is, however, strongly responsive to environmental changes, as prior treatment of the culture vessels with collagen or gelatin on polyvinyl leads to strong accumulation of the factor in the nuclei of the cells. These results argue that the normal growth of Medaka embryonic stem cells is independent of both signalling cascades.

While it was not possible to test for expression of specific STAT3 target genes in Mes1 cells,

the mRNA levels of the major Smad1 target gene id2 was assessable. Real-time PCR quantification of id2 expression in wildtype cells and cells treated with either additional hBMP2 or with the BMP-Smad blocker hBMP2-A34D. This experiment revealed a series of interesting features of Mes1. First of all, there is a readily detectable basal expression level of id2 in untreated Mes1 cells. This level is probably already high enough to prevent low levels of bHLH transcription factors to induce differentiation. This theory also fits to what has been reported for mouse ES-cells and is explained above. It does however, not fit with the exclusively cytoplasmic localisation of Smad4.

Hence the original Medaka orthologue of Smad1 was cloned and fused to GFP. Transfection of this construct clearly shows strong nuclear presence of Smad1 in Mes1 cells. This information suggests a that human Smad4 can not be used as a valid marker for signaling activity in Medaka.

The other parts of the quantitative PCR experiment give contradictory insights into the mechanisms at work: blocking of the BMP-Smad signalling pathway with hBMP2-A34D is not able to lower the mRNA levels of *id2*. Even if this hBMP2 variant was not able to bind BRIa and thus inhibit any downstream signalling, its ability to heterodimerise with wildtype BMP2 would act antagonistically. This finding backs up the cytoplasmic localisation of Smad4 in untreated cells as it proves that cascade inhibition is not translating into transcriptional inhibition and hence the pathway is not the reason for the rather high basal expression level of *id2*. At the same time, the four-fold increase in mRNA levels of *id2* after stimulation with hBMP2 demonstrates several important features of Mes1 cells. On one hand, the BMP2 signalling cascade is present and working in Mes1 cells. On the other hand, *id2* is a direct transcriptional target of BMP2 signalling in Mes1 cells. Additionally, the experiment shows that the human BMP ligand variants used throughout this study are compatible with the Medaka receptors, once more strengthening the importance of BMPs throughout development and hinting at an extremely high evolutionary pressure on the proteins of the pathway; at least structurally. Several explanations for the discrepancy between the localisation studies are feasible:

(1) The human Smad4 protein is, albeit its very high sequence conservation with the Medaka orthologue, not correctly regulated in Mes1 cells, and does therefore not represent the true behaviour of the endogenous Medaka Smad4.

(2) As Smad activator complexes usually exist on the form of multimeric R-Smads and a single Smad4 protein [88], the excessive amounts of Smad4 present in transfected Mes1 cells might lead to cytoplasmic enrichment of Smad4-GPF.

On the other hand, the surplus amounts of Smad1-GFP after transfection could still be efficiently translocatable by the activated pathway.

(3) Even though the Smad1-Smad4 complex is imported into the nucleus, it could dissociate there. Afterwards, Smad1 might induce target gene activation and accumulate in the nucleaus due to its association with DNA and transcription-regulating complexes, while Smad4 could be rapidly exported again.

All three possiblilities are fit to explain the observed phenotypes. For a final clarification of the partially contradictory data described here, it will be necessary to label living Mes1 cells with a nuclear blue fluorescence, mOrange-MFSmad1 and GFP-Smad4 in parallel. These cells should then be filmed over several hours, while being treated with different conditions, such as addition of hBMP2 or hBMP2-A34D and hNoggin. This approach will give insights into the quantitative behaviour of Smad1 and Smad4 in response to different conditions, and would in conjunction with target gene activation studies, help to build a more comprehensive picture of the situation in Mes1 cultures.

Synergistic STAT3 and Smad1 activity is able to maintain mouse embryonic stem cell pluripotency. The presented data indicate both pathways to be inactive in Mes1 cells, hence arguing for a completely different mode of stemness control. However, the in wildytpe cells detectable expression of the Smad1 transcriptional target *id2* – which is mainly responsible for the anti-differentiation effect seen in mouse ES-cells - and its independence of Smad signal blockage opens up a new interpretation option: maybe expression of the Smad1 and STAT3 target genes responsible for the pro-pluripotency effect of both pathways are activated either signal-independently or via different signalling pathways in Mes1 cells. Testing the expression levels of Medaka gene-orthologs of typical stem cell genes such as *oct4* or *nanog* did not give any further insights.

The other tested signalling molecules are all grouped into the MAP-Kinase pathway. They all appear to be active, probably as a result of basic FGF being present in the standard culture medium. Even though the Western blots for phosphorylation levels show differences between Erk1 and Erk2 as well as between Mek1 and Mek2, a quantitative interpretation is not possible because the affinities of the antibodies are not known for Medaka proteins.

3.4 STAT3 during early development

One goal of this study was the comparison of different ES-cell culture systems with their embryonic origin, focusing on the major signalling pathways involved in pluripotency.

Therefore, mouse embryos were fixed at 3.5dpc, the stage commonly used to seed inner-cell-mass (ICM) cells and thus establish ES-cell cultures. Immunofluorescence staining for STAT3 revealed that all cells of the embryos, including the ICM cells, show strong accumulation of the signal transducer protein in their nuclei, suggesting that the pathway is active at this stage of development. It has to be mentioned here, that targeted disruption of the STAT3 gene does not result in a failure of mouse embryos to enter gastrulation, but shows severe patterning phenotypes right after gastrulation [89]. Taken together, these data show that mouse ES-cell cultures correspond to their embryonic origin when considering STAT3 activity status alone, but the functional outcomes of this signal apparently vary.

As discussed before, Medaka ES-cells show exclusively cytoplasmic and thus inactive STAT3 under normal culture conditions. These cells are derived from late blastula embryos. Consequently, immunofluorescence stainings for STAT3 for this embryonic stage were performed. Just like in the cultured cells, STAT3 was always cytoplasmic at this stage of embryogenesis.

These comparative studies show on one hand, that the cell cultures in both investigated species correspond nicely to their embryonic origins with respect to STAT3 localisation. The general difference between the species is striking, though. When considering the relatively late defects of the mouse STAT3 null mutants, the difference could be considered non-influential for the pluripotency state of the early cells in both species. Adding further leverage to this notion are the reports on STAT3 independence of both monkey and human ES-cell cultures [50][49]. Again, in no ES-cell culture system but the mouse, germ line competence, and with that true pluripotency, has been proven to date. Whether the activation of STAT3 does play a role in this context can not be assessed thus far. One option to investigate this would be the generation of a medaka ES-cell line with inducible constitutively-active STAT3. After activation of this transgene for a certain period, inactivation of it followed by direct re-implantation into blastula embryos should result in germ-line contribution of the cells if STAT3 activity is indeed the missing feature.

Interesting are the herein presented results for STAT3 localisation on earlier stages of Medaka embryogenesis by both immunofluoresence and fluorescence-protein tagged MFSTAT3 injection. While STAT3 is exclusively cytoplasmic during the early cleavage stages up to 32 cells, a pattern-free mosaic of cells with nuclear STAT3 is detectable from 64-cell stage onwards. Even more striking is the exclusion of STAT3 from all embryonic cells at 512-cell stage and later. This export borders with the onset of the Medaka mid-blastula transition (MBT), when the zygotic transcriptional programme is activated and

maternal messages are degraded [90][91].

Why a transcription factor such as STAT3 is concertedly imported and then again exported from the nucleus of cells of Medaka embryos before their zygotic transcription starts at MBT is still elusive. It is possible, that STAT3 is required for the initiation of MBT either as an activator of the degradation process of maternal message, or as a factor able to bind to the transcription-repressive molecular, turning them into activators or possible even dragging them out of the nucleus right before MBT starts. While this is pure speculation, corresponding molecular proof may be gathered by a combination of experiments including ectopic expression of constitutively active and dominant negative STAT3 variants coupled with determination of expression timing of genes indicative for MBT.

Furthermore, our understanding of basic signalling principles such as signal strength definition and measurements is still limited. Along with that gap of knowledge come the inability to visualise signals live in a developing embryo in a quantitative way, with minimally invasive techniques. Only with advances in these fields will it be possible to translate our profound knowledge of biochemical interactions into a system-wide understanding of cellular consequences and interplay.

3.5 The physical interaction between STAT3 and BR1a

While the previously described counter-regulation of cell fates mainly executed by target genes of STAT3 and Smad1 is well established, there is but one report on a direct interaction of the transducers of both pathways [12]. Bridged via the transcriptional co-factor p300, the synergistic action of both pathways alters the transcriptional outcome that would be induced by either player alone. A second description of interaction between STAT3 and the BMP pathway shows effects of a BMP ligand on STAT3 phosphorylation at Ser727 [92] in neural stem cells. In this study, a link between BR1a and STAT3 is established indirectly via FKBP12 [93] and the serine-threonine-kinase FKBP12/rapamycin-associated protein (FRAP or mTOR). In these experiments, rapamycin – an inhibitor for FRAP – is able to abolish the phosphorylation of Ser727 of STAT3 upon BMP4 treatment of the neural stem cells. STAT3 was previously shown to be a target of FRAP upon signal-initiation by ciliary neurotrophic factor (CNTF) [94]. However, there is no description of a complex including BMP receptors and STAT3.

Experiments provided in this thesis clearly demonstrate that such a complex exists, and is robustly detectable in a wide variety of cells from different organisms. Subsequently, an

estimation of the size of the complex containing both BR1a and STAT3 was done by HPLC fractionation. The proteins co-eluted in fractions ranging between 70kD, probably the monomeric variants of the proteins, and 800kD – obviously bound to large complexes. The wide range of complex sizes for which both proteins co-elute suggests that they could be core-members of a highly variable complex. The elution fraction would thus depend on the composition of the complex, e.g. the addition of other signalling molecules such as mTOR or PI3K. The fact that STAT3 is mainly found in large cytosplasmic complexes was first reported by Ndubuisi et al. [95].

Linking this and other previous studies to the results presented here, the concept of a widespread existence of a signal-integrator complex for various pathways becomes likely. The existence of such an integration mechanism also opens doors for better explanations for the enormously pleiotrophic effects of many cytokines and morphogens, especially BMPs [70]. Certainly, a more precise mapping of the interaction domains between STAT3 and BR1a needs to follow in order to better understand the architecture of the complex, which will in turn offer further experimental approaches for functional dissection of this crosstalk.

3.6 Functional consequences of the complexation between STAT3 and BR1a

In addition to the finding that BR1a and STAT3 are complexed, starvation experiments of mouse ES-cells followed by treatment with different cytokine combinations revealed that the STAT3 phosphorylation status and also its subcellular localisation are influenced by BR1a ligands.

While mouse ES-cells that were LIF-deprived for six to twelve hours show a significant drop in STAT3-Tyr705 phosphorylation, this residue became again phosphorylated within one hour of addition of either LIF, BMP2 or even BMP2-A34D. In contrast to the general principle of nuclear accumulation of p705-STAT3, immunofluorescence experiments showed a predominantly cytoplasmic distribution of STAT3 after BMP2-A34D addition. Connecting these results with other data on full activation of STAT3, it is possible that the serine727 phosphorylation of STAT3, which is necessary for its full activation [96], is counter regulated by BR1a homodimerisation induced through BMP2-A34D.

Furthermore, activation of STAT3 at residue tyrosine705, which is responsible for dimerisation and nuclear translocation as well as DNA binding, is induced by BRIa binding ligands BMP2 and BMP2-A34D in mouse ES-cells. This later effect has to be an indirect

effect as BMP-receptors only contain serine-threonine kinases.

3.7 Possible mechanisms of the complexation between BR1a and STAT3

Designing functional assays for the crosstalk between two such widely used pathways as described here is far from trivial. Basic gain-of-function and loss-of-function assays aiming at disruption of the interaction will only lead to effects clouded by more pronounced phenotypes arising from general defects of the signalling cascade. To selectively target only this one interaction while leaving the basic linear pathways intact is hardly possible, if technically highly challenging. Currently, only in the mouse system offers the possibility to specifically alter the sequence of the interaction interface of both proteins using through homologous recombination in ES-cells and subsequent generation of transgenic mice.

However, it is clear that at least the effect on the phosphorylation level of STAT3 induced by BR1a ligands is indirect, as BR1a does not have a tyrosine kinase activity. Whether there is a physical interaction between the two proteins as suggested by the finding of two STAT3 binding sites in the C-terminus of BR1a still has to be proven. Then, in a next step, in-vitro assays should be used to determine whether the deletion of these interaction sites translates into abolishment of the STAT3 secondary-modification effect.

Strategies to isolate the kinase responsible for altering tyrosine705 of STAT3 in the here described scenario include a candidate approach using siRNA mediated depletion and mass-spectrometrical sequencing of isolated and purified components of the receptor/STAT3 complex. While candidate approaches seems rather tedious, due to the very high number of potential kinase enzymes able to target STAT3, the biochemical identification of proteins present in the here detected complex will be challenging.

On a mechanistic level, one can only speculate, that BR1a is able to somehow tether phosphorylated STAT3 to the cytoplasm, or even to the membrane. The results using the hBMP2 mutant variant A34D, which is only able to bind BR1a but no type II receptor, suggest that the thus possibly induced formation of BR1a homodimers does influence STAT3 behaviour (*figure* 30).

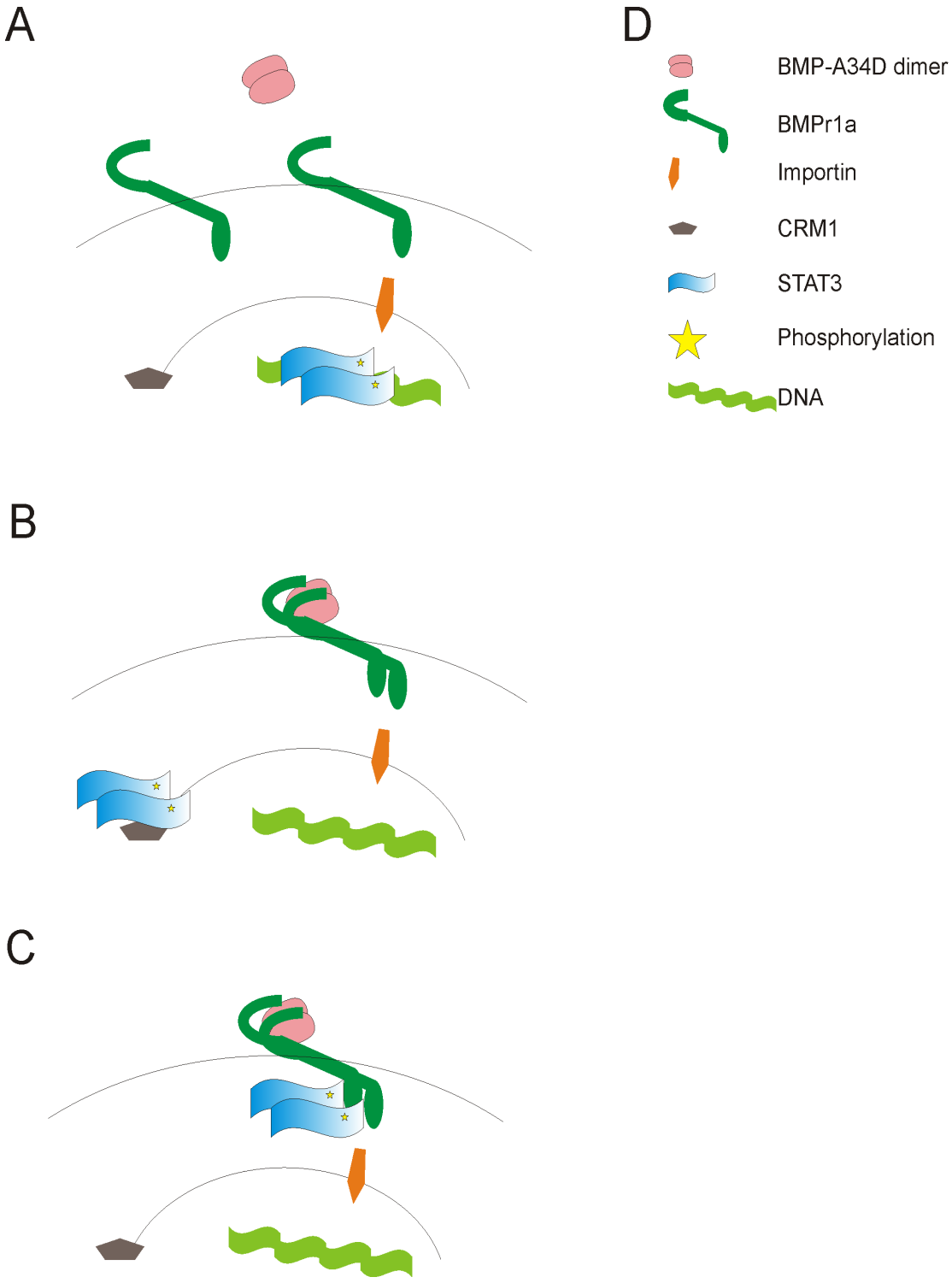

Figure 30: Model for functional consequences of BR1a homodimerisation on STAT3 localisation. Phospohorylated STAT3 homodimers that shuttle between cytoplasm and nucleus (A, B) attach to homodimers of BR1a (C) that are induced by the mutant ligand hBMP2-A34D.

3.8 Outlook

The results described in this thesis represent a basis for further work in the fields of live-signal analysis and evolutionary comparison of molecular stem cell features. Demonstrated is the possibility to localise and quantify signal transducers of the BMP and STAT3 pathways, both very important in many aspects of biology ranging from developmental cell fate decisions to cancer. Using the unmatchable strengths of the Medaka and zebrafish, their transparency, coupled to external development and small size, it will be possible to assess the influence of a wide range of signalling pathways on basically every biological aspect. Thanks to the very high degree of conservation of the molecules involved in signal transduction, the by this approach gained results are potentially translatable to human development and pathogenesis.

The identification of a new branch of BMP signalling, transmitting via modulation of STAT3 localisation and secondary modification, represents a novel mechanism with the potential to investigate and explain observations thus far only seen in the context of both pathways being cytoplasmically isolated. Furthermore, the idea of large signal-integrator complexes is again put forward by the herein described results. It is obvious that the plethora of cellular responses to cytokines can not solely be explained by the mixture of transcription factors in the nucleus. As of late, many pathway intersections, crosstalks, have been reported. Now, with the addition of BMP receptors and STAT3, the possibility of cytoplasmic signal integration and modulation as an additional level of cellular response composition becomes even more plausible.

4. Materials and Methods

4.1 Oligo Sequences

Oligoname	Sequence (5' to 3')
ClonTech-r01	GGACAAACCACAACTAGAATGCAGTGA
ClonTechX1-Sp6-f01	AGCTATTTAGGTGACACTATAGAAGTCAGATCCGCTAGC
cmv_f01	ACGCAAATGGGCGGTAGGCGTGT
EGFP_r02	GGAATTCTCACCATGGTGGCGACCGGTGGA
EGFP-r01	CGGTGAACAGCTCCTCGCCCTT
FLAG-f01	GCTAGCGCTAGCATGGACTACAAAGACGATGACGACAAG
HA-f01	GCTAGCGCTAGCATGTACCCATACGATGTTCCAGATTACGCT
Hsa_BMPrIa-f01	CGCTCGAGATGCCTCAGCTATACATTTACATCAGAT
Hsa_BMPrIa-r01	GCAGAATTCCGATTTTTACATCTTGGGATTCAACCA
hsa_smad1-f01	ACCTAGGCTAGCTCCAAGGAGTATAACTAGTGCTGTCA
hsa_smad1-f02	AATCTCGAGCTATGAATGTGACAAGTTTATTTTCCT
hsa_smad1-r01	GATGCTCTCGAGAGATACAGATGAAATAGGATTATGAGGT
hsa_smad1-r02	CAAGAATTCTTAAGATACAGATGAAATAGGATTATGA
Hsa_smad4-f01	GTCGCTAGCATGGACAATATGTCTATTACGAATA
Hsa_smad4-r01	GTCCTCGAGGTCTAAAGGTTGTGGGTCTGCAATC
MF_ef1a1-f01	GCCCCTGGACACAGAGACTTCATCA
MF_ef1a1-r01	AAGGGGGCTCGGTGGAGTCCAT
MF_FKBP1a-f01	ATGTTACAGAACGGAAAAAAGTTCGACT
MF_FKBP1a-r01	TCACTCCAGCTTGAGCAGCTCCAC
MF_FRAP-f01	GGACACTCCCTGACCCAGTTTGT
MF_FRAP-r01	CCTGGATGAGCATTTTGCGGAGGA
MF_HPRT-f01	CGACCAGTCGACAGGGGAAATCAAAGT
MF_HPRT-r01	GGGTTGTACTGCTTGAGGAGCTCCA
mf_id1-f01	CCTGAAGACCAAGGTCAGCGGAGA
mf_id1-r01	CTGCAAGATTTCCACCTTGCTGGCT
mf_id2-f01	CGAGAATGAAAGCAATAAGCCCTGT
mf_id2-r01	CAGGAGGGACAGCCCCACTTA
mf_id2-r02	CACCAGCTCCTTCAGCTTGGAGTA
MF_nanos-f03	CCCAGGTAGCTTTGAAGTGC
MF_nanos-r03	CGGTACACCATCTCGGACTC
MF_Smad1-f01	AAGCTTCGAATTCTGATTACAAGGATGACGACGATAAGATGAATGTCACCT CACTCTTCTCATTCAC

MF_Smad1-r01	GCCCGGGATCCCTAGGAGACAGAGGATATAGGGTTGT
MF_stat3-f01	CCGGATCCATGGCTCAGTGGAA
mf_stat3-f03	CTTCTTCACCAAGCCTCCTG
mf_stat3-f04	AGGACTCGAGATGGCTCAGTGGAACCAGTTACAGCA
MF_stat3-r01	GGGTAATTTCACATGGGGGAAGCGA
MF_stat3-r02	CAGGAGATTGTGGAACACCAGGGT
mf_stat3-r03	TTGGTGTACGGTTCCACAGA
mf_stat3-r04	CTGCAGAATTCGCTTGTCATCGTCGTCCTTGTAGTCCATGGGGGAAGCG ACGTCCATGT
MF_YY1-f03	TCGAAACCACTATTGTCGGA
MF_yy1-f04	GCACACTTGGGGACAGATACAAACT
MF_YY1-r03	CCCACCACCTCTTCTCTTGT
MF_yy1-r04	ACCTCAACCTCTCTGGTTTGGCCTT
MF_YYI-f02	CGGGATCCTCATGGCGTCCGGGGATACCCT
MF_YYI-r02	GCGCGGCCGCGCCCGTGGCTGAATCACTGGT
mus_aes1-f01	ATGATGTTTCCGCAAAGCCGGCACT
mus_aes1-r01	CTGTAATGCCTCTGCATCTCTGACT
mus_BMPrIa-f01	CGGGATCCCCGCGCGAGACGACGACTGTA
mus_BMPrIa-r01	GCGCGGCCGCTCACCCAGAGCTTTGGGACTGGTCAA
mus_ef1a1-f01	GGTGACAACATGCTGGAGCCAAGTG
mus_ef1a1-r01	CCCACAGGGACAGTGCCAATGC
mus_hprt-f01	CCCTGGTTAAGCAGTACAGCCCCAA
mus_hprt-r01	GGCTTTGTATTTGGCTTTTCCAGTTTCACT
mus_id1-f01	CCCTCCGCCTGTTCTCAGGATCAT
mus_id1-f02	GCCTCAAGGAGCTGGTGCCCA
mus_id1-r01	GTCCCTCAGTGCGCCGCCTCA
mus_id1-r02	CGGCTGGAACACATGCCGCCT
mus_id2-f01	GGTCTCTCCTCCTACGAGCAGCAT
mus_id2-f02	TGCAGATCGCCCTGGACTCGCA
mus_id2-r01	GTCCCCAAATGCCATTTATTTAGCCACA
mus_id2-r02	CCAAGGGCTCTCGCTCGCGCA
mus_id3-f02	CGACCGAGGAGCCTCTTAGCCT
mus_id3-r02	GTTCCGGAGTGAGCTCAGCTGTCT
mus_LifR-f01	CGGGATCCACCATGGGCCAGTAAGCAGCACCCCT
mus_LifR-r01	GCGCGGCCGCTCATGTTTCCTTAATCCATTCTCGCTTCCGAT
mus_nanog-f01	GGATGAAGTGCAAGCGGTGGCAGA
mus_nanog-r01	GCTGCTCCAAGTTGGGTTGGTCCAA
mus_smad1-f01	ACCTAGGCTAGCATGAATGTGACCAGCTTGTTTTCATTCA
mus_smad1-r01	GATGCTCTCGAGGAAAACAGCTCTTCCAAGTCTGCATTGA
mus_smad4-f02	GAGCCCAGGTCATCCTGCTCAC
mus_smad4-r02	CATGCAAGGCGCTCGCACACACT

mus_stat3-f02	ATGAATTCGCAGCAGGATGGCTCAGTGGAAC
mus_stat3-f04	AGACGAATTCAGATCTCTCGAGGAGCTGCAGCAGAAAGTGTCCTACA
mus_stat3-f05	AGACGAATTCAGATCTCTCGAGATGGAGCTGCAGCAGAAAGTGTCCTACA
mus_stat3-r02	GATGGATCCCATGGGGGAGGTAGCACACTC
mus_stat3-r04	CACCATGGATCCCTTATCGTCGTCATCCTTGTAATCTTTCCAAACTGCATCAATGAATGGT
mus_stat3-r05	TCCTGTGCGGCCGCCTTATCGTCGTCATCCTTGTAATCTTTCCAAACTGCATCAATGAATGGT
mus_stat3-r06	TCCTGTGCGGCCGCCTTATCGTCGTCATCCTTGTAATCCTCCAGTTTCTTAATTTGTTGGCGGGT
mus_stat3-r07	TCCTGTGCGGCCGCCTTATCGTCGTCATCCTTGTAATCCCAAAGGGCCAAGATATACTTTTTCACA
mus_stat3-r08	TCCTGTGCGGCCGCTTACTTATCGTCGTCATCCTTGTAATCTTTCCAAACTGCATCAATGAATGGT
mus_stat3-r09	TCCTGTGCGGCCGCTTACTTATCGTCGTCATCCTTGTAATCCTCCAGTTTCTTAATTTGTTGGCGGGT
mus_stat3-r10	TCCTGTGCGGCCGCTTACTTATCGTCGTCATCCTTGTAATCCCAAAGGGCCAAGATATACTTTTTCACAAGGTCGATGATA
mus_yy1-f01	CCTCCTGGAGGGATACCTGGCA
mus_yy1-r01	GCTCTCAACGAACGCTTTGCCACACT
mus-STAT3-f03	AGACGAATTCAGATCTCTCGAGATGGCTCAGTGGAACCAGCT
mus-STAT3-r03	TGGATTGTCGACACCGGTTTACTTGTACAGCTCGTCCAT
musBR1a-f01	GGATCCGAATTCATGACTCAGCTATACACTTACATCAGA
musBR1a-r01	AGATCTCTCGAGTCACTTATCGTCGTCATCCTTGTAATCAATCTTTACATCCTGGGATTCAACCAT
musBR1a-YxxQmut-f01	GAGATCGCCGGCACGAACGGCAAACCTGCAATTGCTCATCGA
musBR1a-YxxQmut-f02	GAGATCGCCGGCACGAAC
musBR1a-YxxQmut-r01	TTTGCCGTTCGTGCCGGCGATCTCTGTGTGGAGGTGGCACAGA
ST3PerfectBSMin-f01	ATCTTGGAATTCTGTGCTTCCCGAACGTTGCTTCCCGAACGTAGATCTGGGTATATAATGGAGCTTGGTACCGAGCTCGGATCCA
ST3ScramblBSMin-f01	ATCTTGGAATTCTGTCTCGTCGACACTGTCTCGTCGACACTGAGATCTGGGTATATAATGGAGCTTGGTACCGAGCTCGGATCCA
STAT3_Seq_f01	GCGTTCAGGACATGGAACAG
STAT3_Seq_f02	TCAGCACCGGCCCGCCCTAG
STAT3_Seq_f02	TCAGCACCGGCCCGCCCTAG
STAT3_seq_r01	ACCAGGATATTGGTGGC
STAT3bind_EcoR1-f01	AGAATTCTGCTTCCCGAACGTGAATTCA
STAT3bind_EcoR1-r01	TGAATTCACGTTCGGGAAGCAGAATTCT
stat3bind-f01	CAGATATCCTCGAGCCCAAGTGCTTCCCGAACGTTGCTTCCCGAACGTAGATCTGGGTATATAA
stat3bind-r01	TGCTTCCCGAACGTTGCTTCCCGAACGTAGATCTGGGTATATAACTCGGATCCAGCCACCATGG
topoM13for	GTAATACGACTCACTATAGGGCGAATTG
topoM13rev	CAGGAAACAGCTATGACCATGATTACG

4.2 Antibodies

Antigen	Dilutions and Blocking	Company	MolecularWeight (kDa)
ß-actin	5% BSA, WB 1:10000	SCBT	43
BRIa	5%BSA, 1:2000 o/n WB or Glycine 1:1000 o/n IF	SCBT	66
FLAG	5% BSA, WB 1:2,000 - 1:10,000 o/n	Rockland	depends on fusion partner
GFP	5% BSA, WB 1:1000	SCBT	depends on fusion partner
phospho Mek1/2	5% BSA, WB 1:1000	SCBT	43 / 30
phospho p38	5% BSA, WB 1:1000	Cell Signalling	38
phospho p42/p44	5% BSA, WB 1:1000	Cell Signaling	42 / 44
phopshoTyr705-STAT3	5% BSA, WB 1:500	SCBT	92
Smad1	5% BSA, WB 1:1000	SCBT	60
STAT3	5%BSA, 1:2000 o/n WB or Glycine 1:1000 o/n IF	SCBT	92

4.3 Cell lines

During the course of the study several mammalian cancer-derived cell lines were used: HeLa, 293T, cos7 and NIH3T3. All these cell lines were kept at 37°C in an incubator with 5% CO2. The medium was composed of DMEM pH7.7, 10% fetal calf serum (PAA Gold 0321) and 1% Penecillin/Streptomycin (Gibco). The murine ES-cell line WW6 was cultured according to Thompson in DMEM, 15% fetal calf serum, 1% Penecillin/Streptomycin, 1xLIF (ESGRO) and L-Glutamine (Gibco), non-essential amino acids (Gibco).

Primary mouse neural stem cells were derived from E14.5 embryos of the CD1 mouse line by Traudel Schmidt. They were kept in basal neuromedium (Gibco) containing 1% Penecillin/Streptomycin, 1% GlutaMax (Gibco), 2% B27 supplement (Gibco), 20ng/ml hEGF

and 20ng/ml bFGF.

The Mes1 cell line was cultured according to Y. Hong in ESM4 medium composed of DMEM, 15% fetal calf serum (PAA GOLD 0321), bFGF, ß-mercaptoethanol, sodium-selenite, non-essential amino acids, L-Glutamine, 1% trout serum, Medaka embryo crude extract (1 embryo per 1ml of medium).

4.4 Special devices

Confocal microscopy: Leica SP2 confocal microscope and corresponding software.

Inverted microscopy: Leica DMI6000 inverted microscope and corresponding software.

RealTime PCR: Bio-Rad iCycler.

Fluorescent Western blot detection: Kodak IM6000.

4.5 Image preparation software

All images were refined for better visualisation with a variety of software: ImageJ, Imaris, Volocity and CorelDraw and Leica Confocal/Microscope Suites. Primary image data was not modified.

4.6 Statistical Analyses

Most statistics were done with OpenOffice Calc (all spreadsheets) and for more advanced graphs SigmaPlot was used.

4.7 Transfection of Mes1 cells

Use 12-wells with MES1 cells at about 60-80% confluence (well growing):

[1] Put 50yl DMEM (pure) into a Eppi

[2] Directly add 1yg Plasmid DNA and mix by pipetting

[3] Leave at RT for 10'

[4] Add 3.5yl Trans-IT LT1 and mix by pipetting 5-10 times

[5] Leave at RT for 30'

[6] wash cells 2x with PBS

[7] put 800yl DMEM-HEPES per well

[8] add the Transfection solution dropwise, do NOT STIR

[9] after 14hours first fluoresence is observable, but weak. With good cells about 50-70% visual

transfection can be achieved

[10] To help the cells recover, add 2x Medium to the well. Keeping the transfection reagent on the cells longer will increase efficiency

4.8 Cell lysis, immunoprecipitation and Western blot

Western blot experiments and immunprecipitations were performed according to the following protocol:

[1] pellet cells (5k-8k 1'-2')

[2] wash 3x in PBS

[3] resuspend in LysisBuffer

LysisBuffer is: 50mM HEPES, 150mM NaCl, 1.5mM MgCl2, 1mM EGTA, 10% Glycerol, 5% Triton-X100, complete before use with: 10µg/ml Aprotinin, 10?g/ml Leupeptin, 200yM Na3VO4, 1mM PMSF, 100mM NaF

[4] keep suspension on ice for 20'

[5] centrifuge 30' 4°C 15k rpm

[6] the lysate can now be frozen at -20°C (usually the supernatant contains the protein) or directly used

4.8.1 Immunoprecipitation

[1] use 400µg of protein lysate

[2] add 1vol (at least 200µl) HNTG Buffer

HNTG Buffer is: 20mM HEPES pH 7.5, 150mM NaCl, 10% Glycerol, 0.1% Triton-X100, complete before use with: 10µg/ml Aprotinin, 10µg/ml Leupeptin, 200yM Na3VO4, 1mM PMSF, 100mM NaF

[3] add 1-2µg of Antibody

[4] add 50µl ProteinA-Sepharose (beads in HNTG w/o inhibitors 1:1volume)

[5] rotate o/n at 4°C

[6] spin down at 15krpm for 5"

[7] wash 3x with 1ml icecold HNTG

[8] remove supernatant except for 30µl, add 7µl 5x leammli, heat to 90°C for 7', load samples

4.8.2 Blotting

[1] take off collection gel

[2] put gel into 1x transfer buffer to equillibrate for 10'

[3] build blot: soft membrane, 2x whattmann, gel, nitrocellulose

[4] membrane, 2x whattmann, soft membrane (everything quite wet)

[5] fill blot with cold transfer buffer and ice-cooler

[6] nitrocellulose membrane side is positive

[7] run at 25mA for 1.5h

4.8.3 Detection

[1] put membrane into TBST for 10'

[2] transfer membrane into blocking solution (usually 5% BSA in TBST w/v) for at least 1h

[3] prepare antibody solution (usually 1:1000 dilution in 5%BSA TBST)

[4] transfer membrane into antibody solution and incubate o/n at 4°C

[5] recover antibody solution and wash membrane at least 7x with 5%BSA in TBST

[6] add secondary antibody at given dilution (1:10000 usually for anti-rabbit HRP) for 1h at RT

[7] wash at least 7x with TBST

[8] develop film using an appropriate kit

4.9 Immunofluorescence

Immunofluorescence stainings were performed according to the following protocol:

[1] wash coverslips in PBS for 5'

[2] fix in 4% Paraformaldehyde in PBS for 10'

[3] wash twice in PBS for 5'

[4] permeabilize in 0.1%TritonX-100 in PBS for 10'

[5] wash in PBS 5'

[6] block in 100mM Glycin, 5% BSA in PBS for 10'

[7] wash in PBS for 5'

[8] incubate with primary antibody solution (1:500 in PBS 5% BSA) for 1h

[9] wash twice in PBS for 5'

[10] block in 100mM Glycin, 5% BSA in PBS for 1h

[11] wash twice in PBS for 5'

[12] incubate with matching secondary antibody for 1h

[13] add Hoechst or DAPI at 1:1000 dilution to the antibody solution for 10'

[14] wash twice in PBS for 5'

[15] prepare a slide with a drop of Mowiol

[16] drop coverslip on Mowiol, keep dark at 4°C and let dry

4.10 RealTime PCR

Per reaction the mix is: 2.5µl 10x buffer, 0.625µl 25mM dNTPs (each is 25mM), 1µl 10pmol primer each, 0.75µl SYBR GREEN Stock (see below), 0.25µl FITC Stock (see below), 2µl cDNA (reaction: 20µl Fermentas Kit + 80µl H2O), 0.2µl Taq Polymerase (15u/µl), 18.675µl H2O

Stocks:

FITC: Bought Stock: 1:10 in DMSO, 0.5yl + 4.5yl DMSO, ad 1ml H2O (only stable for 1 week)

SYBR: Bought Stock: 1:10 in DMSO, 0.5yl + 4.5yl DMSO, ad 1ml H2O (only stable for 1 week)

4.11 PCR

Reactions with a final volume of 20yl were used. Different polymerases (always 1unit) were used together with oligos (1pmol for each), dNTPs (500nM) and Buffer (supplied by the company). As template either plasmids (50ng) or cDNA (varying amounts) were used. Cycling 35 times was standard, but subject to adjustment as necessary.

4.12 Cloning

Plasmids were digested over night with 10U of restriction enzyme in the presence of Shrimp-Alkaline-Phosphatase. DNA fragments to be cloned or subcloned were either amplified with specific primers and subsequently digested or released from donor plasmids by restriction enzymes. Three different molar ratios of target plasmid and insert DNA were mixed, the volume of the reaction was adjusted to 8yl and finally 1yl of 10x Ligase Buffer and 1yl of T4 DNA Ligase (Fermentas) were added. The reaction was kept at room temperature for 1-3 hours and then transformed into chemically competent bacteria.

4.13 Plasmid preparation

Standard alkaline lysis was used in combination with spin-column kits from various companies (QiaGen, PeqLab, Fermentas, Promega) with the supplied protocols.

4.14 mRNA transcription

In vitro transcription of mRNA for injection into fish embryos was synthesized using the mMessage-Machine kit from Ambion according to the supplied protocol.

4.15 Quantification strategy for RealTime PCR

RealTime PCR experiments were analysed using an accepted strategy published and explained online at: http://www.gene-quantification.com/strategy.html .

4.16 Protein alignments

The alignments were done using the free software BioEdit and the included multiple-alignment binary ClustalW (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

5. Contributions by other persons

During the course of the study collaborations with a variety of colleagues contributed significantly to the described experiments. Next to these persons, I would like to thank everybody who helped me over the years, many who I am not able to mention here as their contributions are in projects not represented in this thesis. Following is a wrap-up of major contributions.

Experimental design was done together with Dr. M. Schartl, and refined by discussion with lab members. All contributing work listed below helped with experiments designed by and generally carried out by the author, T. Wagner.

Dr. S. Meierjohann helped with some Western blots and when timed, parallel cell-lysis was important.

MF STAT3 was isolated by T. Wagner and then cloned by M. Kraeussling.

The murine NSC culture was initiated and cells were propagated by T. Schmidt.

The murine stem cell culture was initiated and largely propagated by members of the B. Kneitz lab, especially by C. Reiss.

Extraction of 3.5dpc mouse embryos was done by either L. Federov or C. Reiss.

The HPLC device handling and column calibration were done by A. Chari.

The Mes1 cDNA for some real-time experiments was prepared by E. Thoma.

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7. Summary

The genome of every cell of a vertebrate organism contains the information to build a complete body. Making use of this information is, however, something only a very limited set of cells are capable of. These cells are referred to as pluripotent or totipotent stem cells. During embryogenesis, the single, totipotent cell created by fusion of the two haploid gamete genomes gives rise to billions of more specialised, but also more restricted cells. This restriction is not hardcoded, but subject to modulation - probably controlled through a mixture of chromatin modifications and transcription factor composition.

Hence, the idea that any cell type could first be reprogrammed to become a pluripotent stem cell and then be used for therapeutic tissue replacement. That at least the reprogrammig is possible was lately demonstrated by ectopic expression of four transcription factors (Sox2, cMyc, Klf4, Oct4). Despite this success, the endogenous mechanisms for maintaining or re-creating stem cell status are still elusive.

Describing and analysing naturally used pluripotency controls is therefore of utmost importance. In this thesis, comparison of signalling pathway features between distant vertebrate species with established embryonic stem cell culture systems, mouse and Medaka, was the approach used to study such stemness mechanisms.

In murine stem cells, two signalling pathways play a dominant role for pluripotency upkeep: The LIF-STAT3 transduction system is absolutely essential for prolonged propagation and germ line transmission of the cells, while the BMP2/4-Smad pathway is able to counter-act neural differentiaton allowed by STAT3 activity. Both pathways make use of latent transcription factors for signal transduction. If the pathway is inactive, the transducer proteins are restrained to the cytoplasm and are thus not able to control transcription. On the other hand, ligand-induced activation of the transcription factors leads to nuclear enrichment and target gene activation. Thanks to this mode of action, it was possible to determine the signalling-activity status of both pathways through subcellular localisation studies. For comparing the mouse situation with that in Medaka, both transducer molecules were identified in the genome assembly of the fish and cloned as fusion variants with different fluorescent protein variants. These constructs were then transfected into Medaka embryonic stem (Mes1) cell cultures and imaged live *in vivo*. The results were surprising: while STAT3 is exclusively cytoplasmic and consequently inactive, the transducer of the BMP2 pathway, Smad1, is accumulated in the nuclei of the cells. Next, this culture system

situation was compared to the embryonic origin of the stem cells, the Medaka blastula embryos. In respect to STAT3 localisation, the situation in the embryos was the same as in Mes1 cells, namely completely cytoplasmic. Interestingly, more detailed studies of early embryogenesis revealed that a wave of STAT3 nuclear enrichment occurs between 64- and 512 cell stage, but no activity is detectable during blastula stages.

Thus far, the interplay between BMP and STAT3 pathways was only reported to be at a nuclear level of competition for transcriptional cofactors. During the course of this thesis a direct interaction and complex formation between STAT3 and the BMP receptor 1a (BR1a) was identified. This interaction translated into a secondary modification of STAT3 upon BR1a ligand engagement and furthermore resulted in a subcellular redistribution of STAT3 in mouse embryonic stem cells.

Summarising, the herein presented data suggest that STAT3 is directly influenced by the BMP receptor complex, allowing for high level signal integration between previously isolated pathways. At the same time, STAT3 activation was found to not play a role in either Medaka embryos at blastula stage, nor in the from this stage derived embryonic stem cell cultures. In mouse the situation is different, with STAT3 being active in ES-cell cultures, but also in blastocyst stage embryos. These results, together with the reports on human stem cells being independent of STAT3, suggest that the use of STAT3 in pluripotency control is a peculiarity of mice, and does not represent the evolutionarily basal mechanism.

8. Zusammenfassung

Die DNA jeder Zelle eines Wirbeltiers enthält die Information, die nötig ist, um einen kompletten Körper mit hunderten verschiedenen Zelltypen aufzubauen. Die Fähigkeit, diese Informationen komplett abzurufen, ist allerdings einigen wenigen Zellen des Körpers vorbehalten: den Stammzellen. Während der Embryonalentwicklung entstehen aus der befruchteten totipotenten Eizelle alle anderen Körperzellen, welche sukzessive an Differenzierungspotenzial verlieren. Einige wenige Zellen eines erwachsenenen Wirbeltieres haben noch Stammzellcharakter, sind aber im Gegensatz zu den embryonalen Stammzellen meist auf die Bildung eines relativ kleinen Zelltypsatzes beschränkt. Diese funktionale Einschränkung ist nicht durch genetische Veränderungen fest definiert, sondern unterliegt der Kontrolle von chromatinverändernden Proteinkomplexen sowie der Mixtur an vorhandenen Transkriptionsfaktoren und anderen Regulatoren. All diese Faktoren werden wahrscheinlich durch sogenannte molekulare Nischen, das sind in der Stammzellumgebung vorherrschende Signalstoffgemische, kontrolliert. Was eine Zelle kann, ist also letztlich von den Signalen abhängig, die sie empfängt.

Die vorgelegte Arbeit beschäftigt sich mit einigen Signalkaskaden, die mit der Stammzelligkeit in Verbindung stehen. Ein Großteil der Forschung an Stammzellen erfolgt im Maussystem, welches die besten Methoden zur Kulturetablierung, zur genetischen Veränderung und zur Reimplantation in Embryonen oder adulte Tiere bietet. Wenngleich das Wissen über Stammzellen und deren Eigenschaften stark von dem Maussystem profitiert, ist der isolierte Blick auf ein einzelnes Modell potenziell trügerisch. Dies wurde deutlich, als vor einigen Jahren die Etablierung menschlicher embryonaler Stammzellen gelang. Diese Zellen zeigen stark abweichende Eigenschaften von denen, die für das Modellsystem Maus beschrieben waren. Diese Erkenntnis, verbunden mit der Existenz eines stabilen embryonalen Stammzellmodellsystems aus dem japanischen Reiskärpfling (Medaka, *Oryzias latipes*), ermöglichte den vergleichenden Forschungsansatz, der in dieser Arbeit vorgestellt wird.

Diese Arbeit basiert auf der Annahme, dass eine für Wirbeltiere so basale Funktion wie Stammzelligkeitskontrolle nur bedingt mechanistische Veränderungen toleriert. Daher wurde hier die Aktivität der BMP und STAT3 Signalwege zwischen Medaka-Stammzellkulturen, frühen Medaka-Embryonen, Maus-Stammzellkulturen und auch frühen Mausembryonen verglichen. Interessanterweise konnte festgestellt werden, dass STAT3 zwar in Zellen der Maus- Blastozyste sowie in den Maus Stammzellkulturen aktiv ist,

allerdings inaktiv in Kulturen und Embryonen von Medaka. Dieser Unterschied wird durch Daten aus humanen ES-Zellkulturen unterstützt. Letztere sind ebenfalls komplett STAT3 unabhängig.

Die BMP-Smad Kaskade wiederum ist in Medaka-Stammzellen aktiv, Anti-differenzierungsgene wie *id2*, die durch BMP direkt kontrolliert werden, sind dementsprechend exprimiert. Diese Daten stimmen wiederum mit dem Maussystem überein, während humane ES-Zellen diesbezüglich bislang nicht untersucht wurden.

Die Interaktion zwischen verschiedenen Signalwegen ist ein bisher noch nicht gut verstandenes Gebiet. Die Integration verschiedener Signale ist aber speziell für Stammzellen, die ihr Differenzierungsschicksal von winzigen Abweichungen in der Signalmixtur abhängig machen, von entscheidender Bedeutung. Im zweiten Teil der hier vorgelegten Arbeit konnte eine Interaktion zwischen dem BMP-Rezeptor 1a und STAT3 nachgewiesen werden. Diese Interaktion ist offenbar Teil eines variablen Komplexes. Zum ersten Mal war es auch möglich, funktionale Konsequenzen für STAT3 nach Stimulierung des BMP-Rezeptors 1a zu dokumentieren. Nach Belegung des BMP-Rezeptors 1a mit dem mutierten BMP2-A34D wird STAT3 trotz Aktivierung durch Phosphorylierung an Tyrosin 705 im Zytoplasma von Maus Stammzellen festgehalten.

Zusammengenommen konnte hier gezeigt werden, dass eine Interaktion zwischen den bislang als isoliert betrachteten Signalwegen BMP-Smad und STAT3 besteht. Des Weiteren wurde das Medaka-Stammzellkultursystem benutzt, um zu zeigen, dass STAT3 für die Pluripotenz von Stammzellen nur im Maussystem eine Rolle spielt, wohingegen BMP-Zielgene wie *id2* in bislang allen getesteten ES-Zellkultursystemen aktiv sind.

9. Curriculum vitae including publications

Personal Information

Full Name	Toni Ulrich Wagner
Birthday & Birthplace	12.12.1977 in Bamberg, Germany
Academic Degree	Dipl. Biol.
Private Address	Hessenstrasse 175 97078 Würzburg Germany
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Nationality	German

Education

PhD Thesis in Biology at the University of Würzburg (2003-2007)

University Studies of Biology at the University of Würzburg (1998-2003)

Civil Service at the Zivildienstschule Staffelstein

Primary School and Grammar School (1984-1997)

Memberships

Gesellschaft für Entwicklungsbiologie (German Society for Developmental Biology)

vBio Verband Deutscher Biologen (German Society of Biologists)

International Society for Stem Cell Research

Teaching at University Level

Course assistant for “Biochemistry for Medical Students” (2003-2007)

Supervision of a master thesis (Biomedical Studies, 9 months 2007)

Supervision of a diploma thesis (Biology Studies, 10 months 2006-2007)

Supervision of a bachelor thesis (Biomedical Studies, 3 months 2006)

Supervision of a diploma thesis (Biology Studies, 10 months 2005)

Course assistant for “Developmental Biology Course” (2003-2005)

Supervisor for six 13-week Laboratory Courses (2003-2007)

Fellowships

Course stipend from GlaxoSmithKline for a 1-week practical course on Antibody-Phage-Display at ETH Zurich (by Prof. Neri)

Several travel fellowships as associated member of the Research Training Group 1048 "Organ development in vertebrates": ISSCR Meeting in Boston (2004), StemCellNetwork Meeting in Bonn (2004), GfE School Workshop (2004), Zebrafish Meeting in Dresden (2005), Horizons Meeting in Göttingen (2006)

DAAD fellowship for a 3-month cooperation with the Group of Prof. Scott Fraser, California Institute of Technology, as part of the diploma thesis supervised by Dr. C.Winkler and Dr. R. Köster.

Extracurricular Activities within the University

Elected Student Council (2nd speaker) for the Graduate School of Life-Sciences, University of Wuerzburg

Student representative at the DFG revision of Würzburg's application for the "International Graduate School Würzburg" within the Elite-University Programme.

Co-Head of the organising student-only team of the first "doctoral student-driven biomedical-science conference" in Würzburg 2005.

Elected student speaker of the Research Training Group 1048.

Extracurricular Activities outside of the University

Website Administration using phpBB, phpNuke, Joomla, mySQL along with coding experience in C++, Delphi, Lua, HTML, XML, PHP and SQL.

Youth-Team Coach for Basketball (C-license).

Constant consumption of Audio Books.

Scientific Talks

Invited speaker for a seminar at the GSF in Munich. (2007)

Invited speaker for a seminar at the University of Dresden. (2006)

Student representative speaker at the 1st International Graduate School Symposium Würzburg. (2005)

Repreasentative Speaker at the 2nd Graduate Day of the University of Würzburg. (2005)

Speaker at the regional Fish Meeting Landeck (2004, 2005).

Additional Training

"Das Wissen der Forschung – verständlich für Laien", DFG-Workshop by Dr. Ludwig Kürten, free Science Journalist from Berlin.

"Getting Funded" Workshop on grant applications by BioScript International, Dr. Ruth Willmot.

"Project Management" Workshop by S. Dierig from EinsZeit Managementberatung&Coaching

"Communication Competence" Workshop by F. Tils from ASQ Frankfurt.

"Scientific Presentation" Workshop by BioScript International, Dr. Ruth Willmot.

"Scientific Writing" Workshop by BioScript International, Dr. Ruth Willmot.

"Antibody-Phage Display" practical course at ETH Zurich by Prof. D. Neri.

"Advanced Microscopy" Workshop by Prof. G. Harms & Prof. P. Friedl

Languages

English: fluent, 8 years of grammar school, TOEFL test, 3 months scientific stay in the US, Diploma thesis and PhD thesis written in English, Lab-internal seminars all in English.

French: basic, 5 years of grammar school.

Spanish: basic, ongoing self-teaching.

German: mother tongue.

Computer Skills

Operating Systems: Dos, Windows(95-Vista), Linux (SuSe, Redhat, Fedora Core, Ubuntu), MacOS9-10.

Coding Languages: C++, Delphi, XML, HTML, PHP, mySQL, Lua, Visual Basic.

Office Applications of all sorts, CorelDraw, VectorNTI, SigmaPlot, Microcal Origin & many more.

active Method-Spectrum

PCR, RealTime PCR, Cloning (incl. RecET & SLIC), RNA labelling, Western Blot (ECL & Fluorescent), Immunoprecipitation, Immunofluorescence (cells & embryos), in-situ hybridization (Medaka & Zebrafish), Microinjection (Medaka, Zebrafish &

cultured Cells), Cell culture work (including Virus Production at S2&S1 level), FACS, Phage Display, Microscopy (Light, Confocal, SPIM), computer analyses using numerous online tools (incl. Ensembl&SwissProt).

Scientific Publications

"Bone morphogenetic protein signaling in stem cells – one signal, many consequences"

Wagner TU

FEBS J. 2007 May 29;274(12):2968-2976. Epub 2007 May 22.

PMID 17521338

"Combined loss of Hey1 and HeyL causes congenital heart defects due to impaired epithelial to mesenchymal transformation."

Fischer A, Steidl C, Wagner TU, Lang E, Jakob PM, Friedl P, Knobeloch KP, Gessler M

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