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Review

Novel Growth Network in Muscle Regeneration is Revealed by Molecular Acupuncture-like In Vivo Transfection

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Novel Growth Network in Muscle Regeneration is Revealed by Molecular Acupuncture-like In Vivo Transfection

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Abstract: Low efficiency of in vivo transfection of a few fibers revealed a novel tissue network which temporally amplified growth stimulation in the entire regenerating rat soleus muscle. This acupuncture-like effect was demonstrated when the fibers began to grow after complete fiber degradation, synchronous inflammation, myoblast and myotube formation. The first detection of neonatal sarcoplasmic/endoplasmic reticulum ATPase (SERCA1b) has been made in this system. The neonatal, fast and slow SERCA isoforms displayed consequent change with innervation and differentiation, recapitulating events in muscle development. In vivo transfection of myotubes with plasmids expressing dominant negative Ras or a calcineurin inhibitor peptide (Cain/cabin) proved that the expression of the slow myosin heavy chain and the slow muscle type SERCA2a are differently regulated. In vivo transfection of a few nuclei of myotubes with dnRas or SERCA1b shRNA stimulated fiber size growth in the whole regenerating muscle but only until the full size had been reached. Growth stimulation by Ras and SERCA1b antisense was abolished in co-transfection with Cain or in case of perimuscular injection with IL4 antibody. This revealed a novel signalling network resembling to scale-free networks which, starting from transfected fiber myonuclei as "hubs", can amplify growth stimulation uniformly in the entire regenerating muscle.

Keywords: skeletal muscle; regeneration; molecular acupuncture; SERCA; Ras; in vivo transfection

1. Introduction

Skeletal muscle is a favourite object of differentiation studies. Not just because it is the largest tissue in mammals contributing to around 40% of body weight [1] but also for its importance in movement and healthy life [2]. To maintain integrity of such organ is crucial therefore the skeletal muscle can adapt with remarkable plasticity, be trained, respond to environmental constrains and to pathological conditions [3,4]. The most dramatic response is needed in regeneration when the muscle rebuilds itself after damage [5]. The main source of regeneration are the muscle stem cells singled out from the satellite cells located between the basal lamina and the sarcolemma [6–8]. However, satellite cells behave differently in regeneration than in hypertrophy [9]. In extensive muscle damage bone marrow cells [10–12] and non-muscle-resident fibroblasts [13,14] may also transform to myoblast and contribute to myotubes at the beginning and later during the process. Other stem cells called fibro-adipogenic progenitors can regulate the activity of muscle satellite cells [15,16]. A number of experimental models have been developed for muscle regeneration, but only a few based on complete damage of muscle fibres [17]. Regeneration is more even after complete fibre degradation [18] although the kinetics of changings does not seem to depend on the extent of damage [19]. If fibre degradation is partial the inflammation is sustained by type I macrophages that suppress myoblast formation [20,21]. Experimental regenerations can be induced by local anaesthetics or naturally occurring toxins like snake venoms [22,23]. Bupivacain is probably one of the most often used local anaesthetic for this purpose [24,25] and notexin from the Australian tiger snake [26] is a frequently applied venom. However, notexin induces a more complete muscle damage in mouse tibialis anterior (TA) than bupivacaine [27]. It was also found that rat soleus regenerates with a 1-2 days delay after

intramuscular injection of notexin compared to that after bupivacaine [28,29]. This is because notexin results in a more extensive muscle fibre damage than bupivacaine [27].

In the past few years a significant progress has been made in understanding the mechanism of notexin effect [30]. This A2 phospholipase (PLA2) analogue toxin binds to unidentified receptors on muscle fibres and neuromuscular junctions, it causes sarcolemma damages and starts perturbation that elevates sarcoplasmic Ca2+ level [31,32]. The myotoxic effect is fairly selective for type I and type IIA fibres and apparently spares early differentiated cells like satellite cells, myoblast precursors and myoblasts [18]. The different pathological consequences are probably not due to different mechanisms of cell toxicity but rather to the intrinsic physiological and anatomical properties of the targeted cells [31]. Accordingly, crotoxin, a myotoxin similar to notexin can be antagonised by cyclosporine A (CSA). This can be interpreted so that CSA inhibits opening of the transient permeability pores of the inner membrane of mitochondria [31]. Because oxidative type I and type IIA muscle fibres are more depending on mitochondria than type IIb and type IIx/d glycolytic fibres they are more vulnerable to PLA2 toxins. Notexin can induce muscle necrosis relatively distant from the site of administration but it does not appear to cross blood brain barrier explaining while it could induce muscle regeneration so effectively after subcutaneous injection [33]. Notexin, in contrast to viper venoms [34] do not create extensive haemorrhage since it leaves the blood vessels, the extracellular tissue and the nerves relatively intact, these all act in favour of an even regeneration [35].

Our aim was to establish an experimental system of complete regeneration in order to monitor developmental events. We intend to align the received information with the pattern of in vivo muscle differentiation. Myotube formation and innervation were considered as crucial landmarks. The synchrony of events was studied with morphological, biochemical and molecular biological methods. The whole extract of muscles from each stages of regeneration was studied with practically no contamination of non-regenerating or out-of-stage regenerating tissues. The complete necrosis and subsequent regeneration was achieved by intramuscular injection of ten times higher dose of the purified snake venom (Sigma) than it was administered subcutaneously in earlier reports [33] reviewed in [36]. Such treatment induced a full regeneration process of the soleus muscle of three months old male Wistar rats of 300-360 grams.

2. Muscle degradation, inflammation and increase of TNF-α level

After 6-24 hrs of the intramuscular injection of appropriate amount of snake venom, the soleus became swollen and lost its red colour. This was probably due to the inflammation and the degradation of myoglobin in mitochondria rich fibres that were the most sensitive to the Ca^{2+} entry through the sarcolemma [31,32,37]. The venom toxins bind to a receptor on the sarcolemma and tissue damage is achieved by amplification of the initial effect via signal networks [38]. As the rat soleus muscle contain about 70% type I and 28% type IIA and only 1-2% type IIx fibres [39] this meant practically total fibre degradation. The muscle pallor and weight loss, lasted until day 4-5 of regeneration, then the colour was restored by day 5 but the weight was regained only around day 28 of regeneration [40]. The microscopic changes were the most evident on transversal sections of the necrotised-regenerating muscle; the fibre outline faded already at 6 hours and practically diminished by day 3 after venom administration. A number of mononucleated cells populated at endomysial sites and throughout the cross section of the fibers [18]. In the beginning when the optimal dose of the snake venom was established transversal sections were taken along the spindle shaped soleus scanning from proximal to distal tendons in order to check completeness of muscle degradation. This was also confirmed by the dramatic decline or lack of detection of muscle fibre specific transcripts and proteins (i.e. MyHC-s and SERCA-s, skeletal actin) in the whole muscle homogenate [28,40,41]. However, blood vessels and the endomysia outlines were partially preserved until 3 days after toxin administration [28].

In contrast to the decline of the muscle specific proteins, inflammation markers dramatically increased with the muscle necrosis. The lymphocyte/macrophage specific protein MAC 387 increased after 6 hours showing a maximum on day 3 after toxin injection on immunoblot of whole muscle

extracts [42]. This marker then gradually declined until 28 days after venom injection but still remained above the normal level [42]. The persistence of MAC 387 indicated that lymphocytes/macrophages apparently accompanied muscle regeneration long after necrosis and probably influenced the autocrine-paracrine environment when regeneration looked macro- and microscopically complete [43]. Tumor necrosis factor α (TNF-α), a pleiotropic pro-inflamatory cytokine increased with about two magnitude at the mRNA and protein levels. The comparison of the position of desmin positive muscle cells and the in situ hybridization of TNF-α mRNA on cross sections showed that the cytokine is not expressed in myoblasts and myotubes at day four of regeneration when its level was still around the maximum. The lack of TNF-α expression in muscle myoblasts and myotubes was supported by the absence of its transcript from differentiating in vitro muscle cell cultures like C2C12 and BC3H1 cells however the mRNAs of TNF receptors were expressed in these cell lines. In the in vivo muscle regeneration the change of TNF-α was paralleled by the increase of TNFR-60 but not TNFR-80 mRNA levels suggesting that of the smaller receptor has primary role in the TNF-α response. The role of this cytokine is complex in muscle regeneration. It has been shown that TNF-α acts on muscle cells, just like in other tissues, through more than one pathway [44,45]. One of these pathways at high level of TNF-α, inhibits myogenesis activating NF-χB which translocates to the nucleus and induces iNOS, IL-1 and again TNF-α [46]. The other pathway prevents differentiation through destabilization of myoD [47]. This leads to pro-proliferative and differentiation-suppressing changes [48,49]. The other pathway is through the p38 kinase which phosphorylates essential myogenic transcription factors like MyoD, myogenin and MEF-2 and therefore promotes differentiation to myotubes [50,51]. The later happens at low TNF concentration. TNF-α is also a chemoattractant of satellite cells and mesangioblasts which are able to contribute to regeneration in dystrophic muscle [51,52]. The balance between inflammation and regeneration might be essential for in vivo muscle regeneration [53]. In a preliminary study, we have administered Remicade (cV1q), a drug containing chimeric antibody to TNF-α, subcutaneously and perimuscularly immediately after the venom injection [54]. The Remicade treatment increased fibre size and the intensity of desmin specific immunostaining at 4 days compared to the control regenerating muscle. This supported that TNF-α inhibits early events of muscle regeneration including the formation and growth of myotubes/primitive fibres. This was in agreement with the ameliorating effect of Remicade on skeletal muscle fibrosis in mdx mice, where, however, it also had a negative impact on cardiac function [55]. Myostatin, the first discovered myokine also appeared in this system, according to its growth inhibitory role, suppressed satellite cell activation [56] and later increasingly maintained levels with differentiation [57].
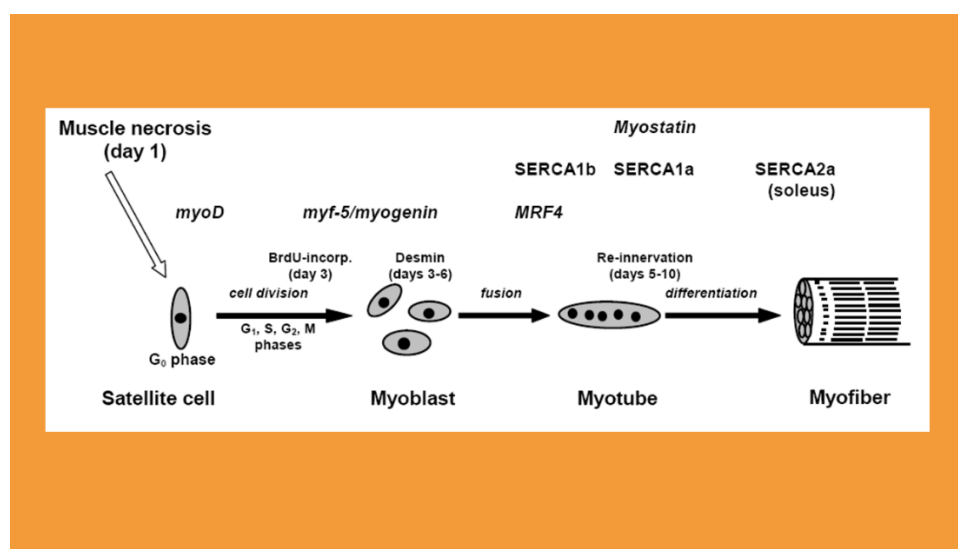
Figure 1. Schematic summary of the studied gene expressions in rat soleus regeneration. The names MRF, SERCA, myostatin, TNF-α indicate the maximum levels of mRNAs/proteins during the characteristic morphological changes of regeneration. Note the consecutive neonatal (SERCA1b), fast

(SERCA1a) and slow type (SERCA2a) calcium pump expressions and that SERCA2a remains the dominant form in the slow type soleus until the end of regeneration. The transfections were done to myotubes formed after for days of regeneration.

3. Myogenic regulatory factors

Skeletal muscle regeneration characteristically includes the selection of cells with mesodermal origin and transforming them into myoblasts [58]. It was a breakthrough in understanding of this process when the myogenic regulatory factors (MRFs), the myoD family had been discovered. The four main members of this family, myoD (myf-3 in human), myf-5, myogenin (myf-4 in human) and MRF-4 (herculin or myf-6) were found to transform fibroblasts or other non-muscle-like cells into myoblasts [59–62]. The MRFs belong to the group of "basic-helix-loop-helix" (bHLH) proteins of transcription factors that bind the E-box (CANNTAG) with their basic domain when form homodimers or heterodimers with each other or (in most cells) with non-myogenic E proteins [63–65]. Indeed, the E-box is present in the regulatory region of a number of muscle specific genes like the alpha subunit of nicotinic acetylcholine receptor, the myosin light chain, alpha-actin, desmin, troponin I, and creatine kinase and needed for muscle specific expression [66–68]. The different role of MRFs can be assumed according to their spatial and temporal expression and the diverse phenotype of their knock-out mutants [61,69]. The specificity of MRFs can be find in pursuit of interactions of their transactivation domain with other transcription factors. MyoD and myf-5 are playing important role in transforming muscle precursor cells of the somite into myoblasts during early embryogenesis of mammals by activating chromatin loci of muscle specific genes [67,70–72].

It was of interest to describe the mRNA levels of myogenic regulatory factors in muscle regeneration and as far as we know our work was the first to do this [18]. Originally we aimed to find homology between muscle regeneration and embryonic myogenesis and reveal differences in slow (soleus) and fast (EDL) muscle regeneration. In soleus regeneration, as it were expected, the myoD increased first on day one and reached a maximum on day 3 then gradually declined to near to normal level by days 21. The myf-5 and myogenin mRNA levels also declined on day one then increased to maximum on day 3 and decreased gradually. On day one the decline of MRF4 mRNA was the most dramatic compared to the other factors and to the normal muscle level and became restored the latest by day 5 of regeneration. This showed that MRF4 is more bound to the myotubes/fibers than to myoblasts. Interestingly the EDL muscle regeneration did not show much differences in changes of myoD, myf-5 and myogenin mRNA levels compared to the soleus. However the MRF4 was not declining significantly as in the soleus most likely because the EDL regeneration was less synchronous and had many not degraded/partially damaged large fibres (probably IIb and IIx types) [18]. Besides this the timing of expression of myogenic factors at the resolution of our work was largely similar to the order (myoD/myf-5, myogenin, MRF4) reported in myogenesis [68,73]. A remarkable difference was in the level myf-5 mRNA which increased in myogenesis at about the same time as the myoD message did [63] but it declined in our regeneration system in contrast to myoD. It has been reported that the myoD knock-out mice showed a slightly delayed but otherwise quite normal muscle development. This could happen because the increased myf-5 expression with the same cell specificity complemented for myoD function [69]. Such an altered regulation of myf-5 and myoD might happen in muscle regeneration as well. The other explanation for the decreased myf-5 message level might be that the satellite cells expressing myf-5 [74,75] were transformed into muscle progenitor cells and myoblasts and from that time expressed predominantly myoD.

The myoD protein followed its message in the regenerating soleus and showed a 6x increase compared to the normal level, a maximum on the 3rd day after venom injection. This was paralleled by the maximum level of BrdU incorporation in to the nucleus implicating that the proliferating cells, probably myoblasts expressed the transcription factor. We interfered with the myoD expression by injecting antisense oligonucleotides into the muscle on the 3rd day of regeneration [76]. The antisense decreased the mRNA and protein level of myoD dramatically at one hour after the treatment; the myoD protein was about 3-fold less in the isolated myonuclei compared to the control regenerating soleus injected with scrambled oligonucleotide. The progress of regeneration was also delayed by

this treatment as it was judged two days later by desmin expression, myotube formation and the number of primitive endplates [76,77]. This was somewhat a surprise since myoD was reported to have a relatively quick turnover in cell culture [78,79]. Apparently our antisense oligo started to degrade after four hours and the myoD mRNA level was also restored by that time [76].   However the antisense still worked probably because it hybridized to the translation start of myoD [80] and contained a TCC sequence that enhanced the antisense effect by stimulating RNase H activity [81]. The antisense effect resulted in other interesting changes; the mRNA levels of myoD myf-5 and myogenin were elevated again after 24 hours probably to bring in the backlog of regeneration. However the mRNA levels of MRF4 and the myogenic factor inhibitory proteins Id1 and Id3 did not change. In the subsequent period no apparent difference was observed in the myoD antisense treated muscles except that after 28 days more small and split fibres were reflecting the delayed regeneration [76]. The above results clearly showed that myoD has a crucial role of in muscle regeneration and indicated that this system can be a tool for investigating similar functions of the other myogenic factors [82]. The expression of connexins have also been screened in notexin induced in vivo muscle regeneration by others. They found that only connexion 43 gap junction protein (Cx43) is present and it is upregulated transiently before myoblast fusion [83]. Dye injection of in vitro primary muscle cells revealed more intense gap junction coupling between sparsely located pre-fusion myoblasts than between the close fusion aligned myoblasts, suggesting that this tight junction protein takes part in synchronisation of cell cycle control and preparation for fusion to syncytial cell [84]. Transfection of skeletal muscle primary cultures with dominant negative Cx43 confirmed that activity of this protein is needed for myoblast proliferation and syncytial fusion into myotubes [85]. Later it has been found that in regenerating muscle the cluster of differentiated satellite cells which represent an advanced stage of fusion in the inner part of the basal membrane do not express Cx43, suggesting that this protein does not participate actively in myoblast fusion although it is required for preparation to that process [86].

4. The expression of sarco/endoplasmic reticulum Ca2+ ATPases (SERCAs) in regeneration

The SERCAs are a group of transmembrane proteins that pump the Ca2+ from the cyto/sarcoplasm to the endo/sarcoplasmic reticulum. In striated muscle this creates a situation in which the troponin complex can occupy the actin binding place of myosin head and prevent actomyosin formation [87]. This has an impact in aging and various pathological conditions [88]. There are three strongly homologous SERCA genes (SERCA1-3 or ATP2A1-3 according to the human nomenclature) but only two of them, SERCA1 and SERCA2 have splice variants expressed in striated muscle. SERCA1a is responsible for relaxation in adult fast skeletal muscle, SERCA1b is expressed in neonatal muscle and SERCA2a is found in slow skeletal muscle and cardio myocyte in heart ventricle [89–91]. The mRNA of SERCA1b was detected mostly in neonatal skeletal muscle and our work was the first to demonstrate that the protein is highly specific to myotubes and developing fibers but not transforming or adapting muscles [92]. We also showed that SERCA1b is a major isoform in regenerating (fast or slow) muscle but not expressed in a healthy adult rat or adult human muscle where otherwise its splicing could be several-fold increased i.e. by denervation or stretch [92–97]. We also found in accordance with others [98,99] that the SERCA1b protein was expressed in a pathological condition of adult human muscle called myotonic dystrophy type II [96]. Nevertheless, although the regenerative potential was up-built in ischemic lower limb muscle of atherosclerosis obliterans no SERCA1b only SERCA1a was upregulated [97].

In accordance the neonatal 1b is the first SERCA isoform that appears shortly after the myogenic regulatory factors in regenerating rat soleus and EDL muscles [28,41,100]. This predominant expression of SERCA1b indicates myotube formation and it is the forerunner of the orchestrated pattern of SERCA isoforms found characteristically at both message and protein levels in muscle regeneration. This motif is strongly dependent on the progress of innervation, the type of innervation (fast or slow) and it is expressed in developing myofibres before they become fast or slow. This means that the start of innervation will switch the splicing of SERCA1 transcript from neonatal to adult fast type mRNA in both fast and slow muscle regeneration [28,100]. The switch between SERCA1a and

SERCA2a occurs in slow fibre regeneration when innervation is more established [77,101] and the fast SERCA1a declines while the slow SERCA2a elevates becoming exclusive (or at least predominant) [28,41]. Interestingly the fast twitch muscle follows an alternate motif which starts with a moderate elevation then decline of SERCA2a expression, paralleled with those of SERCA1b before the increase and predominance of SERCA1a while the innervation progresses [100].

The change of SERCA levels appears to be similar to the innervation dependent expression of myosin isoforms in regeneration especially in the predominantly slow soleus muscle [33,102]. This prompted us to investigate if the slow muscle type SERCA2a level is co-regulated with that of the heavy chain of corresponding slow myosin isoform, MyHC1. In bupivacaine induced regeneration of rat soleus the MyHC1 expression is upregulated by slow type innervation [29]. The upregulation can be prevented by transfecting dominant negative Ras N17 (dnRas) into the regenerating muscle fibres [29]. Interestingly when bupivacain regeneration was induced in denervated rat soleus the transfection of Ras V12 S35, which influenced downstream the MAPK pathway, restored the MyHC1 expression in the fibres while the other Ras mutants did not [29]. This suggested that a distinct well defined pathway mediates the nerve effect on MyHC1 expression in regeneration. We wondered if the same pathway could also function in upregulation of SERCA2a. However, we found that the expression of SERCA2a was not dependent on slow type innervation like the MyHC1 neither could be influenced by transfection of Ras mutants regulating MyHC1 expression [40]. Therefore the slow type of SERCA2a and MyHC1 are not co-regulated by innervation and Ras. Similar conclusion was drawn after experiments with calcineurin [103]. The calcineurin-NFAT pathway was also found to mediate the effect of slow type innervation on the expression of MyHC1 in bupivacaine induced regeneration of rat soleus [104,105]. However, transfection with Cain, while it prevented MyHC1, it did not prevent the expression of SERCA2a in the notexin induced regeneration of rat soleus [103]. This again confirmed that the SERCA2a expression is regulated by separate pathway(s) from those controlling slow myosin expression. Later it was reported that slow myosin do not depend neither on slow type innervation nor on calcineurin in other muscle than the soleus [106]. However we have not found a similar report for SERCA2a. The question remained: what regulates then SERCA2a expression? First of all SERCA2a regulation is not exerted so much on the transcript level as the MxHC1 expression [29] but clearly has more components [107]. One of these complex effects is the passive stretch that works in selective denervation of soleus but not in ischiadic (hindlimb) denervation. To illustrate this, SERCA2a was upregulated in the selective denervated vs. ischiadic denervated regenerating soleus [40]. Similarly SERCA2a was upregulated in dnRas transfected fibres of innervated regenerating muscle which obviously were stretched by the overwhelming innervated fibres [40].

5. A novel muscle growth regulation revealed by molecular acupuncture in regeneration takes after scale-free network

The transfection of regenerating soleus with dnRas resulted in a phenomenon that according to our knowledge has not been reported yet [108]; besides inhibiting expression of slow MyHCI in the transfected fibre [29] dnRas resulted in a non-fiber-autonomous effect. Although only 20-30 fibres were transfected out of the total (approximately 2500), expressing the transgene in a few of their myonuclei [109], the fibre size (cross sectional area, CSA) was increased in the entire muscle between days 7-12 of regeneration compared to the control regenerating soleus transfected with the empty vector [108]. The size of transfected fibres was not different from the non-transfected ones within the same muscle and showed no morphological or histochemical difference. However, after 21 days when the full fiber size was nearly reached, the growth increase was attenuated therefore it did not exceeded that of the control. The augmentation of fibre growth must have started after transfection on day 4 and was finished by day 21 of regeneration. and was apparently mediated by autocrine/paracrine effect. One of the factors that might orchestrate the effect of transfection was interleukin 4 (IL4) since the perimuscular injection of its antibody stopped the augmentation of CSA increase. However, the IL4 antibody did not decreased fibre size below the level of the non-transfected regenerating soleus suggesting that the cytokine has a role in augmentation of fibre

growth rather than in the normal regeneration process. Interestingly the higher IL4 level was detectable only around the transfected fibres by immunohistochemistry and not in the whole muscle by immunoblot supporting local importance instead of a wide spread systematic one. The IL4 is secreted by growing myotubes and acts on receptors of myoblast stimulating their accretion [110,111]. As the IL4 expression is regulated by the calcineurin-NFAT pathway [110,112] the calcineurin inhibitor peptide cain/cabin was co-transfected with dnRas in the regenerating soleus [108]. This prevented the augmentation of CSA increase suggesting that the calcineurin-NFAT-IL4 was activated by Ras inhibition. This revealed a scenario in which the calcineurin-NFAT and Ras pathways act in balance in controlling fibre growth of regenerating soleus muscle. According to this scene Ras kept calcineurin under inhibition but the transfected dnRas gave a way for the increase of calcineurin activity and growth augmentation.

Although Ras and calcineurin are members of two important pathways to our knowledge there are little data about their connection in skeletal muscle. However, in neonatal cardiac myocytes their interaction appears synergistic not antagonistic as in the regenerating muscle since the constitutively active Ras increased the activity of NFAT3 [113]. Ras acted upstream of calcineurin on NFAT3 activity, and similarly, dominant negative Ras inhibited the stimulated NFAT nuclear location and transcriptional activity [113]. Essentially similar coordinated functions have been found in vivo between calcineurin-NFAT and the Ras stimulated MAPK-ERK pathway in cardiac hypertrophy of transgenic mice [114]. Since this interaction has not been enlightened in muscle cells we silenced the main pump that regulates sarcoplasmic $[Ca^{2+}]_i$ in regenerating muscle fibres. SERCA1b is a major isoform in neonatal and regenerating muscles [92,96,98]. Transfection of SERCA1b shRNA expressing vector into fibres of regenerating soleus resulted in augmentation of CSA increase in both transfected and non-transfected fibres of the whole muscle [115] in a similar manner to that of dnRAS [108]. The growth stimulation was also calcineurin and IL4 dependent as it was abolished by co-transfection with Cain and the perimuscular injection of IL4 antibody [115]. This indicated the pivotal role of the calcineurin-NFAT-IL4 pathway in both dnRas and SERCA1b silencing induced fiber growth augmentation of regenerating soleus muscle.

It is worth to emphasize that silencing of SERCA1b in a small fraction of fibers had dramatically different effect from that of knocking out of SERCA1 gene in mice as the later resulted in perinatal lethality [116]. However, it showed a somewhat similar phenomenon to the muscle specific knock out of SERCA inhibiting micropeptide myoregulin which in mice resulted in enhanced exercise performance [117].

Remarkably, only a few (1-4) myonuclei had been transfected per fiber in the muscle belly [109]. The total number of myonuclei in a regenerating fiber can be estimated as several fold higher than the ones having been transfected [109,118]. Therefore, the growth stimulation must have been initiated from a small minority of myonuclei [108,109,115]. However, the myonuclei surrounded by zones form myonuclear domain which is a functional units of gene expression within the multinucleated fiber [119]. The plasmids have to reach the nuclei therefore transfect the myonuclear domain in order to get expression. Such rare transfection of nuclei like it happened in the regenerating rat soleus is resembling to the effect of acupuncture [120]. However, this time the manipulation was made by molecular inhibitions such as the dominant negative Ras or SERCA1b silencing instead of a special needle. So this phenomenon can be described illustratively as a molecular acupuncture of regenerating skeletal muscle. Furthermore, if one wants to depict the relationship of transfected and non-transfected myonuclear domains in the above described stimulated fiber growth, it is appealing to adapt a scale-free network at least for the initial events (Figure 2) [121,122]. In this network the nodes are not identical since some of them have higher number of links than most of the rest has. These high-degree nodes become hubs compared to the other low-degree nodes which are in abundance. The hub quality is gained by transfected myonuclei because of their calcineurin activity while the non-transfected nuclei remain low-degree nodes. The regenerating muscle is probably self-organizing network and create new hubs from low-degree nodes by other features as none of the transfected fibers showed hypertrophy compared to the non-transfected others. Independent regulation of myonuclei for gene expression has been shown along multinucleated fibers and

suggested to be organized in stochastic pattern [123]. However, according to my knowledge, no network like interaction between myonuclear domains in different myofibers has been reported. This also suggests that the regeneration system discussed here may fill a gap in the study of biological networks.
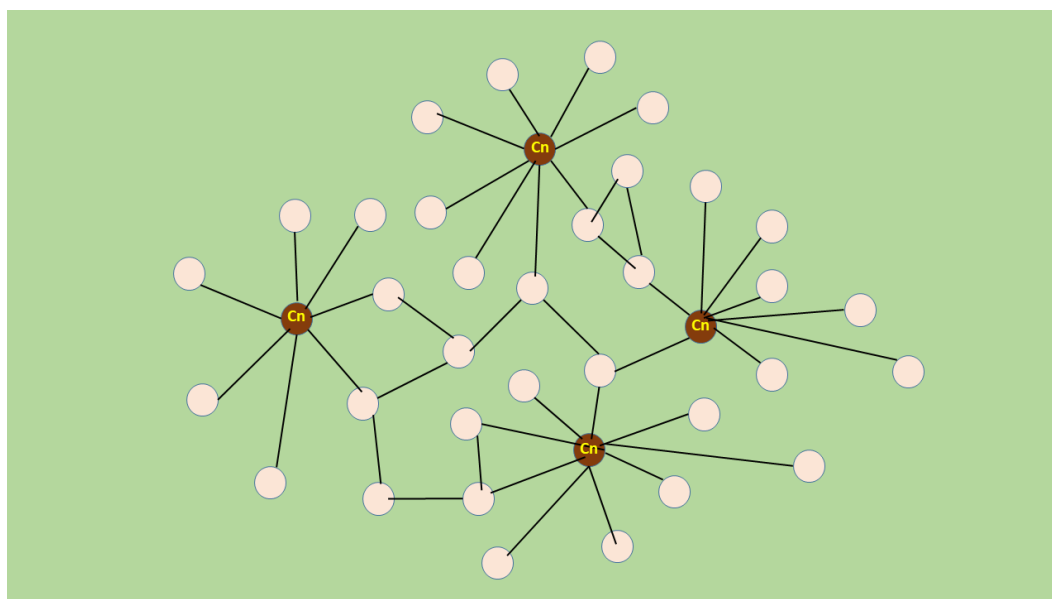
Figure 2. Scale-free network in molecular-acupuncture-stimulated muscle regeneration. Myonuclear domains (Md) are depicted as nodes (illustrated with circles) and lines indicate links between the nodes. Md transforms into „hub" (a high grade node with many links) when transfected with dnRas or SERCA1b shRNA probably because of its increased calcineurin (Cn) activity. The links between hubs and nontransfected Mds might be formed by autocrine-paracrine factors like IL4. Cn indicates Md with hub-creating calcineurin activity. This representation does not intend to show the entire operation of the growth network, it only meant to reflect the driving process.

6. Conclusions

A synchronized in vivo muscle regeneration revealed a novel network of growth regulation. The network is stimulated by changing the equilibrium between two major signal transduction systems, controlled by Ras and calcineurin, within the same muscle fibre. Shifting this dynamic balance, by stimulating calcineurin, influences other fibers probably, in autocrine-paracrine way and augments fiber growth in the entire regenerating muscle. The reason that such an effect has not been reported before might be that the growth stimulation was not possible to detect reliably in unsynchronized and partial regenerations where the fibers did not grow evenly therefore comparison with the control was hampered. So the notexin induced soleus regeneration in rat offers a unique in vivo system for studying novel effects of signal pathway interactions and apparently represents an example of scale-free networks of signalling in skeletal muscle. It would be interesting to see whether other co-transfections with dnRAS could interfere with SERCA1b shRNA induced fibre growth amelioration and if the transfection alone with calcineurin inhibitor Cain could have any effect. Experiments in this direction are on their way.

9

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